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**THE ROLE OF ENDOPLASMIC RETICULUM IN
HUMAN ADIPOSE TISSUE**

By

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A thesis submitted to
The Faculty of Medicine
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DEDICATION

To my parents for their encouragement and prayers.

And to my wife and daughters for enduring the hardship of the journey.

DECLARATION

I declare that this thesis is an accurate record of my results obtained by myself within the labs at University of Warwick, Clinical Science Research Institute and, the data that has arisen is detailed in this thesis. All sources of support and technical assistance have been stated in the text of the acknowledgments. None of the work has been previously submitted for a higher degree.

All sources have been specifically acknowledged by means of reference.

Summary

Adipose tissue plays a central role in the regulation of metabolic homeostasis. In obesity adipocytes are challenged by many insults: surplus energy, inflammation, insulin resistance and considerable endoplasmic reticulum (ER) stress. ER stress has been casually linked to increased inflammation and insulin resistance. Also, obesity linked type 2 diabetes is associated with hyperglycaemia, lipotoxicity and endotoxemia. Therefore, the aims of this thesis briefly were to 1) characterise human pre-adipocytes during differentiation, as a suitable primary cellular model to examine intracellular pathways, 2) investigate the role of glucose and fatty acids on ER stress pathway; as these primary insults are considered to have clear impact on inflammation, insulin resistance (IR) status and diabetes pathogenesis 3) to examine the role of lipopolysaccharide (LPS), a gut derived bacterial fragment, on ER stress; as LPS is now considered a systemic circulating factor raised in conditions of IR, 4) the role of salicylate, known to have anti-inflammatory properties which may negate or at least attenuate the effects of ER stress.

Components of the ER stress pathways were studied in human abdominal subcutaneous (AbSc) adipose tissue (AT) from obese and lean subjects. Following characterisation, culture and differentiation of primary human pre-adipocytes, these adipocytes were treated with lipopolysaccharide (LPS), high glucose (HG), tunicamycin (Tun) and saturated fatty acids (SFA) either alone or in combination with sodium salicylate (Sal). Quantitative RT-PCR, western blotting, adipokine analysis were used to assess expression levels.

Markers of ER stress were significantly increased in AbSc AT from subjects with obesity ($P<0.001$). Differentiated primary human adipocytes treated with LPS, Tun, HG and SFA showed significant activation of p-eIF2 α and ATF6 and their downstream targets ($P<0.05$). This effect was alleviated in the presence of Sal. There was also significant activation of AktSer473 during ER stress ($P<0.05$).

This thesis presents important evidence that firstly, there is increased ER stress in human adipose tissue of obese individuals, secondly, LPS, hyperglycaemia and saturated fatty acids induce significant ER stress in primary human adipocytes and finally that induction is alleviated by salicylate. Taken together these studies highlights that ER stress occurs in human differentiated pre-adipocytes is exacerbated in conditions of high glucose, high saturated fatty acids and LPS, as well as determining that such primary insults can be reduced by salicylates providing initial evidence that therapeutic agents have the potential capacity to alleviate ER stress in human adipose tissue.

ABBREVIATIONS

AbSc	Abdominal Subcutaneous
AbSc AT	Abdominal Subcutaneous Adipose Tissue
Ad	Adipocyte
AMPK	AMP-activated Protein Kinase
ANCOVA	Analysis of Covariance
ANGII	Angiotensinogen II
AP-1	Activator Protein - 1
ap2	Adipocyte Fatty Acid Binding-protein
ASK-1	Apoptosis signal-regulating kinase -1
AT	Adipose Tissue
ATF-4	Activating transcription factor - 4
ATF-6	Activating transcription factor - 6
ATF-6f	Activating transcription factor-6 fragment
ATP	Adenosine tri phosphate
BAT	Brown Adipose Tissue
Bip	Binding immunoglobulin protein
BMI	Body Mass Index
BSA	Bovine Serum Albumin
C	Control
CEBP-α	CCAAT enhancer-binding protein- α
CHD	Coronary heart disease
CHO	Chinese hamster ovary cell line
CHOP	Cyclophosphamide hydroxydaunorubicin oncovin prednisone
CRP	C-reactive Protein
Ct	Cycle Threshold
CT	Computed tomography
CV	Coefficient of Variance
CVD	Cardiovascular Disease

Da	Daltons
ΔCt	Delta Cycle Threshold
DGAT-2	Diacylglycerol acyltransferase-2
DHA	Decosahexanoic acid
dH₂O	Distilled Water
DMEM	Dulbecco's Minimum Essential Medium
DMSO	Dimethylsulphoxide
DNA	Deoxyribonucleic Acid
DNase	Deoxyribonuclease
dNTPs	Deoxynucleotides Triphosphates
DTT	Dithiothreitol
DEXA	Dual-emission X-ray absorptiometry
eIF2α	eukaryotic Translational Initiation Factor 2 α
ECL	Enhanced Chemiluminescence
EDTA	Ethylenediaminetetraacetic Acid
ELISA	Enzyme-linked Immunosorbant Assay
EMBL-EBI	European Bioinformatics Institute
EPA	Eicosapentaenoic acid
ER	Endoplasmic Reticulum
ERAD	Endoplasmic Reticulum associated protein degradation
Ero-1	Endoplasmic Reticulum oxidoreductin-1
FABP-2	Fatty Acid Binding-protein-2
FABPs	Fatty Acid Binding-proteins
FAM	RT-PCR Reporter Fluorochrome/Dye Label
FDG	Fludeoxyglucose
FFAs	Free Fatty Acids
FGF	Fibroblast Growth Factor
FPLC	Fast Protein Liquid Chromatography
<i>g</i>	Force of Gravity
GI	Gastrointestinal
GLUT-4	Glucose-transporter-4

GLUTs	Glucose Transporters
G6Pase	Glucose-6-phosphatase
Grp78	Glucose-regulated protein 78 kDa
GSK-3	Glycogen Synthase Kinase-3
HBSS	Hank's Balanced Salt Solution
HDL	High density lipoprotein
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic Acid
HG	High glucose
HMW	Higher Molecular Weight
H₂O	Water
HOMA	Homeostasis Model Assessment
HOMA-IR	Homeostasis Model Assessment of Insulin Resistance
hr	Hour
HRP	Horseradish Peroxidase
HSL	Hormone Sensitive Lipase
IBMX	3-Isobutyl-1-Methylxanthine
ICAM-1	Intercellular Adhesion Molecule-1
IDF	International diabetes federation
IFG	Impaired fasting glycaemia
IGF-1	Insulin-like Growth Factor-1
IGF-1BP	IGF-1 Binding protein
IGT	Impaired Glucose Tolerance
IκBα	Inhibitor of NF- κ B - α
IKK	Inhibitor of NF- κ B Kinase
IKKα	Inhibitor of NF- κ B Kinase- α
IKKβ	Inhibitor of NF- κ B Kinase- β
IKKγ/NEMO	Inhibitor of NF- κ B Kinase- γ
IL	Interleukin
IL-1	Interleukin-1
IL-1β	Interleukin-1- β
IL-6	Interleukin-6
IL-8	Interleukin-8
IL-11	Interleukin-11

IOTF	International obesity task force
IR	Insulin Resistance
IRS	Insulin Receptor Substrate
IRS-1	Insulin Receptor Substrate-1
IRS-2	Insulin Receptor Substrate-2
JNK	c-Jun N-terminal Kinase
kDa	Kilodaltons
kg	kilogram
L	Litre
LMW	Low Molecular Weight
LPS	Lipopolysaccharide
M	Molar
M_r	Molecular Weight
MAPK	Mitogen-activated Protein Kinase
MCF-7	Michigan Cancer Foundation – 7 cell line
MCP-1	Monocyte Chemotactic Protein-1
MgCl₂	Magnesium Chloride
MGP	Matrix gla protein
MRI	Magnetic resonance imaging
MS	Metabolic Syndrome
µg	Microgram
m²	meter squared
mg	Milligram
min	Minute (time)
µl	Microlitre
ml	Millilitre
mM	Millimolar
MMPs	Matrix Metalloproteinases
MONICA	Multinational Monitoring of Trends and Determinants in CVD
MPO	Myeloperoxidase
MUFAs	Mono-saturated fatty acids

mQH₂O	Milli Q water (ultra-filtered water)
mRNA	Messenger Ribonucleic acid
NaCl	Sodium Chloride
NF-κB	Nuclear Factor-κB
NEFA	Non-esterified Fatty Acid
NHS	National health service
NIK	NF-κB-inducing Kinase
N	Number
ng	Nanogram
nm	Nanometre
NPY	Neuropeptide Y
N.S	Non-significant
<i>ob/ob</i>	Leptin-deficient Mouse
OD	Optical Density
ODU	Optical Density Unit
Om	Omental
Om Ad	Omental Adipocytes
Om AT	Omental Adipose Tissue
p	Phosphorylated
PAI-1	Plasminogen Activator Inhibitor-1
PBA	Phenylbutyrate
PBS	Phosphate-buffered Saline
PBS-T	Phosphate-buffered Saline containing 0.1% Tween 20
PCR	Polymerase Chain Reaction
PDI	Protein disulphide isomerase
PEPCK	Phosphoenolpyruvate Carboxylase
PERK	Protein kinase RNA-like endoplasmic reticulum kinase
p-eIF2α	phospho-eukaryotic initiation factor 2 α
p-PERK	phospho- protein kinase endoplasmic reticulum kinase
PET	Positron emission tomography
PDK-1	3-phosphoinositide-dependent Protein Kinase-1
PI3K	Phosphoinositide-3 Kinase
PKB/Akt	Protein Kinase B

PKC	Protein Kinase C
POMC	Proopiomelanocortin
PPAR-γ	Peroxisome Proliferator Activated Receptor- γ
PPG	Postprandial hyperglycemia
PPI	Peptidyl Proline Isomerase
PPREs	PPAR- γ Response Elements
psi	Pounds Per Square Inch
PUFA	Polysaturated fat
p-value	Probability value
PVDF	Polyvinylidene-fluoride
RBP-4	Retinol binding protein-4
RIA	Radioimmunoassay
RIPA	Radio-Immunoprecipitation Assay
RNA	Ribonucleic Acid
ROS	Reactive Oxygen Species
RSG	Rosiglitazone
RT	Room Temperature
RTn	Reverse Transcriptase
RT-PCR	Real-time PCR
s	Second (time)
S1P	Site 1 protease
S2P	Site 2 protease
Sal	salicylate
Sc	Subcutaneous
Sc Ad	Subcutaneous Adipocytes
Sc AT	Subcutaneous Adipose Tissue
Ser	Serine Residue
SD	Standard Deviation
SDS	Sodium Dodecyl Sulphate
SDS-PAGE	SDS-polyacrylamide Gel Electrophoresis
SEM	Standard Error of the Mean

SFA	Saturated fatty acid
SNPs	Single Nucleotide Polymorphisms
SPSS	Statistical Package for the Social Sciences
SVF	Stromal vascular fluid
Taq	Thermus Aquaticus (DNA polymerase)
TBS	Tris-buffered Saline
TBS-T	Tris-buffered Saline containing 0.1% Tween 20
TCA	Trichloroacetic Acid
T2DM	Type 2 Diabetes Mellitus
TEMED	N, N, N', N'-Tetramethylethelenediamine
TFAs	Trans fatty acids
TG	Triglyceride
TGF-β	Transforming Growth Factor- β
TLR	Toll-like Receptor
TLRs	Toll-like Receptors
TNF-α	Tumour Necrosis Factor- α
TNFR	TNF- α Receptor
TRAF	TNF Receptor-associated Factor
TRAF-2	TNF Receptor-associated Factor-2
Tris	Tris (hydroxymethyl) Aminomethane
Tris-HCl	Tris Hydrochloride
TUDCA	Tauroursodeoxycholate
Tun	Tunicamycin
TZDs	Thiozoladinediones
U	Units
UCP-1	Uncoupling protein-1
UK	United Kingdom
UPR	Unfolded protein response
USA	United States of America
UV	Ultraviolet

VAT	Visceral adipose tissue
V	Volts
VIC	RT-PCR Fluorochrome/Dye Label
v/v	Ratio of Volume per Volume
w/v	Ratio of Weight per Volume
WAT	White Adipose Tissue
WHO	World Health Organisation
XBP-1	X-box binding protein-1
XBP-1s	X-box binding protein-1 spliced
yrs	Years

Chapter 1

Introduction

1.1 Definition and Epidemiology of Obesity

Obesity is defined as an excessive accumulation of fat that may impair health (WHO). According to the World Health Organisation there were 400 million obese adults in 2005 and it is estimated that there will be more than 700 million obese adults by 2015. Due to the widespread of obesity across the world, obesity is now considered to have reached pandemic proportions (pandemic being the widespread of obesity across geographical locations (WHO, 2006)).

Within the UK population, obese individuals account for 23% of total population in 2003 (NHS, 2010). By 2007, the UK Department Office for Science published a report, FORESIGHT REPORT, addressing the prevalence of obesity in the UK. In this report the scientific panel estimated that 40% of Britons will be obese by 2025 which will make Britain an obese society by 2050 (Butland *et al*, 2007). However such obesity is not limited to the UK, the world's largest economy, United States of America, has shown an alarming increase in obese population since the 1960's (NHLBI, 2000). With a population around 240 millions the latest published reports estimate obese population to be over one third of Americans, 33.8 %, as such this currently suggests just under 73 million people are managing with obesity daily (Flegal *et al*, 2010). Indeed, obesity is such a worldwide problem that it is not just affecting the western world but emerging economies such as China, India as well as the developing countries in the Middle East. In 2002, data showed that obese individuals comprised about 2.6 % of the Chinese population (Wu, 2006). Although, the percentage may appear small, its translation into 26 million of individuals which will have future significance in the biggest populated nation on earth. Currently, WHO estimates obesity levels in China lie just under 5%. This is an alarming increase in obesity levels coming from a nation once considered to have the leanest population. India is

also following suite and obesity is acknowledged as a rising health risk indicator (Agrawal, 2002). As per 2006 reports, obesity rates range between 12.1 % and 14.8 % for men and women respectively (IIPS, 2006). Surprisingly, countries in the Middle East are claiming their territory on the obesity map as well. A study published in 2005 suggested that an overall obesity prevalence of 35.5 % in the Kingdom of Saudi Arabia (Al-Nozha *et al*, 2005) whilst the United Arab Emirates, although small in size and population also reported high prevalence of obesity (Malik & Bakir, 2007) The epidemic of obesity is so global that it has proven that no region of the world is spared. Even those considered poor African countries that are classified as under-nutritioned nations are being hit by the obesity epidemic. It has become apparent that obesity now co-exists alongside under-nutrition in the African continent (Kruger *et al*, 2005).

1.2 Consequences of Obesity

As is the case with any ailment, obesity is a health condition that bears medical, psychological and economic consequences. The following sections will look at these aspects in more detail

1.2.1 Medical

The scientific evidence is mounting rapidly of the medical consequences associated with obesity. It has been shown that obesity is linked to cardiovascular diseases such as heart conditions and strokes (Poirier *et al*, 2006; Lavie *et al*, 2009; Arsenault *et al*, 2010). Obesity predisposes to diabetes (Mokdad *et al*, 2003; Lazar, 2005; Dandona *et al*, 2005; Freemantle *et al*, 2008) and is associated with musculoskeletal disorders such as osteoarthritis (Wearing *et al*, 2006; Krul *et al*,

2009; Anandacoomarasamy *et al*, 2008). Some medical research has also linked obesity to the development of some cancers such as endometrial, colon and breast cancers (Rapp *et al*, 2005; van Kruijsdijk *et al*, 2009; Fleming *et al*, 2009; Sinicrope & Dannenberg, 2011; Roberts *et al*, 2010).

1.2.2 Psychological

Obese individuals are often stigmatised and it has been often socially acceptable to become the centre of ridicule. Even the modern media and film-industry play a part in promoting this social attitude towards obesity. These social pressures manifest themselves in the form of psychological disorders suffered by the obese individuals. These disorders range from low-self esteem and social isolation from the rest of the community to clinical depression. Psychologically, self-isolation and clinical depression often prelude to increased food intake as a compensatory measure for loneliness which creates a vicious cycle that revolves around obesity (Friedman *et al*, 2002; Pratt *et al*, 2007; Gariepy *et al*, 2010).

1.2.3 Economic

The world has started to realize the economic and financial implications of obesity. The world health organization and governments of the industrialized world are feeling the burden of the increasing requirement to deal with obesity associated diseases; all of which presents a developing substantial burden in order to either treat current conditions or long-term look into prevention programmes. In England, the economic cost of treating obesity and its associated

ailments was estimated to be over four billion pounds in 2007 (Butland *et al*, 2007; Swanton, 2008) whereas the estimates in the United States of America were over 75 billion dollars in 2000 (CDC, 2009) and both estimates are rising annually (Sassi *et al*, 2010).

1.2.4 Global Intervention

In response to the realisation of

- a) the medical, social and economic implications of obesity and
- b) its epidemic proportions

Many countries have created different organizations, societies and research centres to address the wide spread of obesity and find practical solutions to its associated problems. Indeed, obesity has become a major and urgent issue for the International obesity task force (IOTF). This organisation was established to provide guidelines and propose policies to combat obesity (IASO, 2011). Such guidelines examined adult populations and their needs as well as looking long term to examine the needs and potential prevention requirements for children to reduce the developing obesity epidemic in this group. Childhood obesity has been increasingly reported in many countries, whether industrialised or developing, and these countries highlight the rise and the concern for the next generation as childhood obesity develops widely (Malik & Bakir, 2007; Ji, 2008; Jafar *et al*, 2008; Gill *et al*, 2009; Sullivan, 2010; Stamatakis *et al*, 2010).

1.3 Measurement of Obesity

Epidemiological studies which were carried out just after World War II concluded that the best practical formulae to reflect the range of body weight indices was dividing the weight in kilograms by the square of height in meters [Weight (kg) / height (m²)]. This ratio was called the Quetelet Index after Adolphe Quetelet (1796 – 1874), he was a Belgian social scientist and statistician who pioneered this kind of epidemiological studies. However, Quetelet Index was changed into Body Mass Index (BMI) by Ancel Keys in 1972 (Eknayan, 2008) which has become familiar to most people as a way of categorising body weight.

Classification	BMI(kg/m ²)
	Principal cut-off points
<i>Underweight</i>	<i><18.50</i>
✓ Severe thinness	<16.00
✓ Moderate thinness	16.00 - 16.99
✓ Mild thinness	17.00 - 18.49
<i>Normal range</i>	<i>18.50 - 24.99</i>
<i>Overweight</i>	<i>≥25.00</i>
✓ Pre-obese	25.00 - 29.99
<i>Obese</i>	<i>≥30.00</i>
✓ Obese class I	30.00 - 34.99
✓ Obese class II	35.00 - 39.99
✓ Obese class III	≥40.00

Table 1.3.1 Categorising humans based on body mass index into different groups,

underweight, normal range, overweight or obese classes. (www.who.int/bmi)

Due to the broad range of body weights, the world health organization has divided body mass index into categories as shown in the table 1.3.1.

It is important to note that body mass index, waist circumference and waist-hip ratio can give a relatively clear index of potential risk by using such simple measurements. Primary care physicians find such measurements easy to determine from patients and monitor; noting caution that this is not to determine muscle mass to fat mass ratio. However such assessment of adipose tissue only gives an approximate idea concerning general total body fat; more accurate measurement of adipose tissue mass and distribution can be obtained by performing sophisticated analytical procedures such as Computed Tomography (CT), Dual Energy X-ray Absorption (DEXA) and Magnetic Resonance Imaging (MRI) (Bosello & Zamboni, 2000; Bozzetto *et al*, 2010; Kuk *et al*, 2005; Gallagher *et al*, 2009).

1.4 Diabetes

Obesity predisposes to many conditions including type 2 diabetes Mellitus (T2DM). According to the world health organisation, T2DM is defined as “a chronic disease that occurs either when the pancreas does not produce enough insulin or when the body cannot effectively use the insulin it produces” (WHO, 2011). Insulin aids target tissues such as liver, fat (adipose tissue) and muscle, to uptake glucose from the blood stream for metabolism. Depending on the cause, diabetes is classified into three different types as follows:-

- I. Type 1 diabetes where the pancreas does not produce sufficient insulin for blood glucose metabolism. This type of diabetes can only be controlled by life-long and daily administration of insulin.
- II. T2DM where there is sufficient amount of circulating insulin in the blood but the target body tissues are not responsive enough to the circulating insulin. Controlled management of this type of diabetes ranges from physical activities and diet to insulin sensitizers and agonists.
- III. Gestational diabetes is defined as a state of increased blood glucose during pregnancy. This type of diabetes is pregnancy-related and can be cured if managed properly during pregnancy.

There are also conditions which prelude to diabetes such as impaired glucose tolerance (IGT) and impaired fasting glycaemia (IFG); these two states form intermediaries, where blood glucose levels are bordering the cut-off points, between normality and diabetes. People with impaired glucose tolerance and impaired fasting glycaemia are at higher risk than normal people in becoming diabetic in later life.

1.4.1 Prevalence of Diabetes

T2DM is the most common form of diabetes comprising about 90% of diabetics. According to the current estimates, there are more than 220 million diabetics worldwide and the number is rising annually (WHO, 2011). In England, the prevalence of diabetes is about 5.4% in 2009 (NHS, 2010) whereas its prevalence in the United States of America is estimated at about 7.8% in 2007 (NIDDKD, 2011). Globally, the prevalence of diabetes show surprising results being

most prevalent in developing countries. The ten top countries of diabetes prevalence lie in the Middle and Far East regions of the world (IDF, 2006).

1.4.2 Complications of Diabetes

As T2DM progresses, it starts affecting different body tissues. Therefore, its range of complications is wide and varied. Complications of T2DM have been well established (Reenders *et al*, 1993; Fowler, 2008; Mazzone *et al*, 2008; Moore *et al*, 2009). Patients with Diabetes have much higher risk of developing cardiovascular diseases (Kannel & McGee, 1979; Fox *et al*, 2007; Kelly *et al*, 2009), late stages of diabetes can lead to neuropathy (Caputo *et al*, 1994; Resnick *et al*, 2002; Mert *et al*, 2010), retinopathy (Kahn & Bradley, 1975; Bodansky *et al*, 1982; Moloney & Drury, 1982; Fong *et al*, 2004) and nephropathy (Borchjohnsen *et al*, 1992; Parving *et al*, 2001; Schena & Gesualdo, 2005).

In neuropathy, the patient with T2DM starts losing sensation and feeling in limbs, most commonly feet, which may end up in amputation of the limb; in diabetic retinopathy, the patient's eye sight begins to deteriorate until blindness; and in nephropathy, progressive diabetes affects the functionality of the kidney resulting in kidney failure eventually. It is evident that late stages of T2DM can be fatal. However, fatal consequences of T2DM can be triggered at any stage of diabetes if it is uncontrolled such as in the case of developing ketoacidosis. Subjects with T2DM are also higher vulnerability to infections. It is also worth noting that patients with T2DM are prone to psychological depression as well (Lin *et al*, 2010).

1.4.3 Global Impact

Due to the increasing prevalence of T2DM and the wide range of complications associated with it, world economies expenditure on treatment and prevention is totalling billions of dollars (Zimmet *et al*, 2001; Yach *et al*, 2006). Worldwide there are many organisations and societies formed in response to the urgent need for advisory and research bodies to promote diabetes prevention and improve its treatment (IDF, 2006).

1.4.4 Obesity-Induced Diabetes or Diabesity

The link between obesity and diabetes is evident as research progression indicates (Astrup & Finer, 2000; Freemantle *et al*, 2008). This close link prompted some researchers to coin a new term elucidating this link, diabesity (Astrup & Finer, 2000; Farag & Gaballa, 2011). Although, obesity is a risk factor for developing T2DM, it should be noted that not all obese subjects are diabetic nor will develop diabetes at later stage. As mentioned earlier that obesity is a risk factor for CVD as well, as such morbid obesity coexists with heart attacks, strokes, hypertension and T2DM. These associated factors led to the existence of a cluster of pathologies collectively termed metabolic syndrome or syndrome X (James *et al*, 2004; Weiss *et al*, 2004; Bruce *et al*, 2011; Han & Lean, 2011). World health organisation set five defining criteria for diagnosing metabolic syndrome. Those criteria were slightly simplified by the international diabetes federation so that they become more practical and could be accommodated for use in the clinics by physicians (**Table 1.4.4.1**).

<p>Criteria for diagnosis of metabolic syndrome:</p> <ol style="list-style-type: none"> 1. Central obesity (waist circumference of ≥ 94 cm for European men and ≥ 80 cm for European women, with ethnicity specific values for other groups)
<p>In addition to any TWO of the following four factors:</p> <ol style="list-style-type: none"> 2. Raised triglyceride level: (≥ 150 mg/dL or 1.7 mmol/L) Or specific treatment for this lipid abnormality 3. Reduced high density lipoprotein, HDL: (<40 mg/dL or 1.03 mmol/L in males) and (<50 mg/dL or 1.29 mmol/L in females) Or specific treatment for this lipid abnormality 4. Raised blood pressure: (systolic BP ≥ 130 or diastolic BP ≥ 85 mm Hg) Or treatment of previously diagnosed hypertension 5. Raised fasting plasma glucose, FPG: (≥ 100 mg/dL or 5.6 mmol/L) Or previously diagnosed type 2 diabetes

Table 1.4.4.1 The new International Diabetes Federation (IDF) definition, 2011

The recommendation from the IDF was that three out of the five criteria should be present in a patient for the condition to be diagnosed as metabolic syndrome. However, one single criterion is central to the diagnosis of metabolic syndrome, namely increased body waist circumference; meaning this criterion must always be one of the three factors (James *et al*, 2004). This emphasis on abdominal obesity proves the instrumental role played by obesity compared with other morbidities; one of which is T2DM. An increase in waist girth has been shown to be a valid indicator of morbid obesity and a risk factor associated with other diseases such as CVD and T2DM, hence the current emphasis on the measurement of the increase in body waist circumference. Another important aspect of measuring the waist circumference is its practicality

and ease of its use in the clinic (Grundy, 2004; Despres & Lemieux, 2006; Yoshimura *et al*, 2011). Obesity-induced diabetes has been the theme of research for the last decade to elucidate the mechanisms by which it can develop. The current answer to the question of how obesity causes T2DM directs us to the core of obesity, the role of adipose tissue. The widely accepted explanation is that chemical messengers secreted by adipose tissue interfere with the action of insulin in the target tissues leading to a phenomenon called insulin resistance (Bosello & Zamboni, 2000; Weiss *et al*, 2004; Despres & Lemieux, 2006; Han & Lean, 2011).

In this condition, target tissues such as liver, muscle and adipose tissue itself all become insensitive to the normal level of circulating insulin in the bloodstream. This results in increased level of glucose in the blood and glucose starvation in the target tissue which responds by synthesising glucose, gluconeogenesis as is the case in of liver and muscle. In addition, liver cells secrete glucose into the blood stream which increases the glucose level even further, thus creating a vicious cycle. Adipose tissue in itself is a target tissue for insulin and insulin resistance in adipose tissue which may induce even more detrimental consequences on health such as hypertriglyceridemia and ectopic adiposity. Hypertriglyceridemia adds a compounding effect to insulin resistance by interfering with insulin action at the cellular level. Ectopic adiposity is the deposition of fat outside the adipose tissue such as liver, muscle, heart and kidneys. Ectopic adiposity occurs when the adipose tissue is overloaded and excess fat is transferred to other sites for deposition. Ectopic adiposity exacerbates the pathologic effects of adipose tissue as it compromises the functionality of the other tissues as TG are not supposed to be stores in these organs (Bosello & Zamboni, 2000; Yoshimura *et al*, 2011). For these reasons adipose tissue is the focus of ongoing research.

1.5 Adipose Tissue

For many years, adipose had been regarded as the storage site of excess energy in the form of triglycerides. During prolonged postprandial fasting those triglycerides are broken down into free fatty acids and metabolised to produce energy units, Adenosine Tri Phosphate (ATP). The free fatty acids are secreted into the bloodstream to be metabolised by the liver as well. Another function of adipose tissue is to act as a physical cushion from external physical bodily insults and a thermal insulator from cold. Adipose tissue plays a more active role in the body thermoregulation where it produces heat in a process called thermogenesis. The main component of adipose tissue is adipocytes (Figure 1.5.1). Nonetheless, it contains other cell types which are collectively referred to as the Stromal Vascular Fraction (SVF). Stromal vascular fraction consists of cells such as pre-adipocytes, mature adipocytes, vascular cells, nerves, macrophages and fibroblasts (Gesta *et al*, 2007).

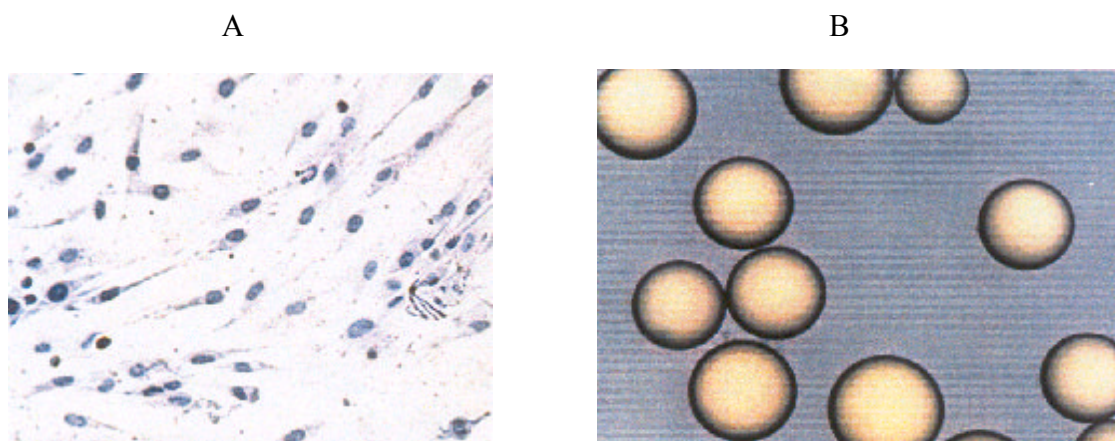


Figure 1.5.1 This shows human (panel A) pre-adipocyte cells during proliferation and (panel B) mature lipid filled adipocytes. (Magnification x20)

There are two types of adipose tissue: white adipose tissue (WAT) and brown adipose tissue (BAT). These two types are distinctive both in terms of histology and function. The reference to brown fat occurs due to colour distinction based on the morphological appearance of adipocytes (Figure 1.5.2). Brown adipocytes contain much more mitochondrial organelles, thus appearing brown when viewed under the microscope (Gesta *et al*, 2007). WAT is unilobular and considered to acts as a storage facility for free fatty acids (although also has a multitude of other important endocrine functions regulating appetite and insulin sensitivity through release of adipokines discovered over recent years). In contrast, the main function of BAT is to generate heat for the organism through non-shivering effects of uncoupled oxidative metabolism, utilizing Uncoupling Protein 1 (UCP-1). Histologically, BAT appears multilobular and contains an abundance of mitochondria, providing its brown coloration.

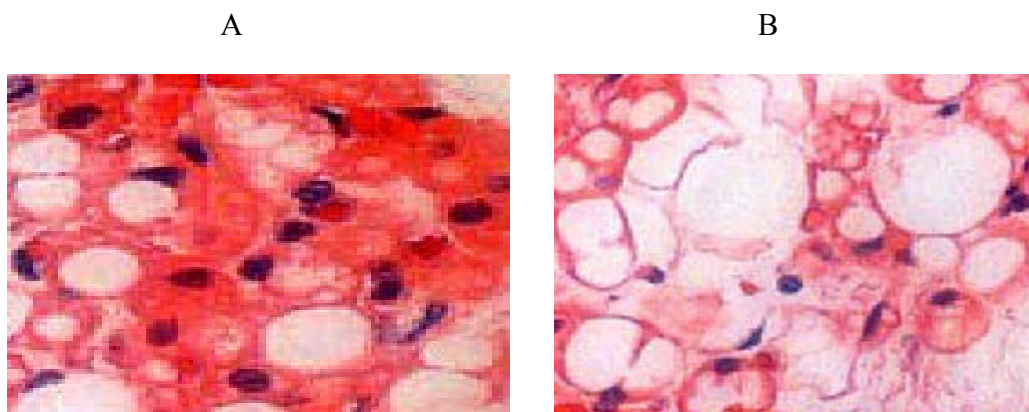


Figure 1.5.2 Histological comparison of human (A) brown adipose tissue showing dense intracellular matter observed with red staining and (B) white adipose tissue showing mature (lipid filled) adipocyte cells without dense intracellular matter (denoted by a honeycomb appearance). (A, B: x 20 magnification).

Due to its function as heat-generating tissue, BAT also has a rich blood supply to improve the efficiency of heat transport around the organism (Enerback, 2010a; Enerback, 2010b). Due to their relatively large surface area to volume ratios, BAT developed evolutionarily in small mammals as a means to avoid reductions in body temperatures resulting from relatively large losses of heat from the skin. Human neonates also contain a relatively large amount of inter-scapular BAT which is presumably an adaptive response to a proportionately large amount of heat dissipation from the skin of human neonates (compared with human adults).

There has been a resurgence of interest in BAT in recent years following the discovery that human adults (in addition to human neonates) also have some BAT. There is also evidence that the *activity* of BAT (shown on Positron Emission Tomography Computerized Tomography Fluoro-Deoxy-Glucose [PET/CT FDG] uptake scans) in human adults correlates with age, BMI and sex with more BAT occurring in young and lean women (Pfannenberger *et al*, 2010; Lee *et al*, 2010). There is even evidence that BAT activity (shown on PET/CT FDG uptake) varies in the *same* individual over time, and that this variance in BAT activity also correlates with longitudinal changes in BMI in the same individual (Lee *et al*, 2010). Potential seasonal changes in BAT activity, independent of changes in BMI, could be of further interest. From a research perspective, the presence of BAT in adult humans is interesting as BAT activity, through the generation of heat, expends energy in the process and therefore holds the potential to affect metabolic rate and promote weight loss. In addition to appetite and exercise, a further novel approach to managing obesity might be through manipulation of BAT and therein lies an important goal for the future. Despite its clear relevance for human metabolism, and its potential for therapeutic manipulation, a major obstacle to the study of human BAT to date has been the lack of a safe, reliable, accurate, sensitive and reproducible means of quantifying the amount of

BAT present through techniques such as imaging and other biochemical biomarkers. Without such techniques, it is difficult to derive meaningful conclusions about the role of BAT in humans

Research into the genetic profile of adipose tissue found some difference in gene expression between BAT and WAT. Site specific variations in genetic gene expression have also been shown within white adipose tissue itself; that is subcutaneous verses visceral (Gesta *et al*, 2006; Vohl *et al*, 2004) highlighting adipose tissues depots at times as unique sites.

1.5.1 Abdominal Subcutaneous & Omental Adipose Tissue

In human adults, white adipose tissue can be found in several anatomical sites and depending on the site, white adipose tissue is termed either as subcutaneous (Sc) adipose tissue or Visceral (V) adipose tissue. As the terms imply, subcutaneous adipose tissue is located under the skin layer mostly in thighs, buttocks, arms, face, neck and back region whereas visceral adipose tissue lies deep within the viscera such as omentum, intestines and perirenal areas (Gesta *et al*, 2007; Bozzetto *et al*, 2010; Kuk *et al*, 2005). Abdominal subcutaneous adipose tissue can comprise as much as 80% of total adipose tissue mass, with Visceral adiposity only comprising 6-20% of the total adipose tissue mass (Montague *et al*, 1997). Therefore changes in adipose tissue distribution, as well as alteration in circulating factors related to risk profile, can have critical influences on adipose tissue metabolism. From our anatomical understanding, omental adipose tissue drains directly into the portal vein and further studies have suggested (Klötting *et al*, 2007) that this exposure of the liver to high concentrations of metabolites and adipokines released from visceral adipocytes may increase the cardio-metabolic risk (Hayashi *et al*, 2004; Onat *et al*, 2007; Despres *et al*, 2001; Empana *et al*, 2004; Elisha *et al*, 2010).

It should also be documented that similar studies have also addressed the risk of accumulating abdominal subcutaneous adipose tissue, (Blackburn & Waltman, 2005; Sardinha *et al*, 2000; Goodpaster *et al*, 1997; Misra *et al*, 1997) whilst all conclude that central obesity remains a reliable predictor of these cardiovascular disease profiles (Lee *et al*, 2007; Valsamakis *et al*, 2004; Smith *et al*, 2006; Nordstrand *et al*, 2011; Murakami *et al*, 2007).

1.5.2 Gender Differences in Adipose Tissue Distribution

Differential anatomic accumulation of adipose tissue is fundamental to the metabolic risk profile of T2DM. It is apparent that different patterns of obesity occur; central obesity is noted as an increase in intra-abdominal fat, particularly abdominal subcutaneous and visceral adipose tissue. Lower body obesity however is characterised by fat stored predominantly in subcutaneous regions of hips, thighs and lower trunk. These different fat distributions are readily identified between women and men, as we note men to be more apple shaped and women to be noted as more pear shaped in their adipose tissue distribution, this upper body obesity (*i.e.* central or abdominal) is also referred to as either 'android' or 'male type obesity' and is compared with 'gynoid' or 'female type obesity' (lower body or gluteo-femoral) identified in women. It should be stressed that age and BMI matched women have a lower incidence of T2DM than men, however changes in fat distribution during the menopause have been shown to correlate with a higher incidence of glucose intolerance and high fasting plasma triglycerides compared with women with predominantly lower body or 'gynoid fat' distribution (Kissebah *et al*, 1982); which offers an important marker for potential metabolic complications.

In addition to the increased hyperlipolytic activity in omental (visceral) versus abdominal subcutaneous adipose tissue which has been implied to mediate profound differences in receptor expression and adipokine secretion between subcutaneous and visceral adipocytes.

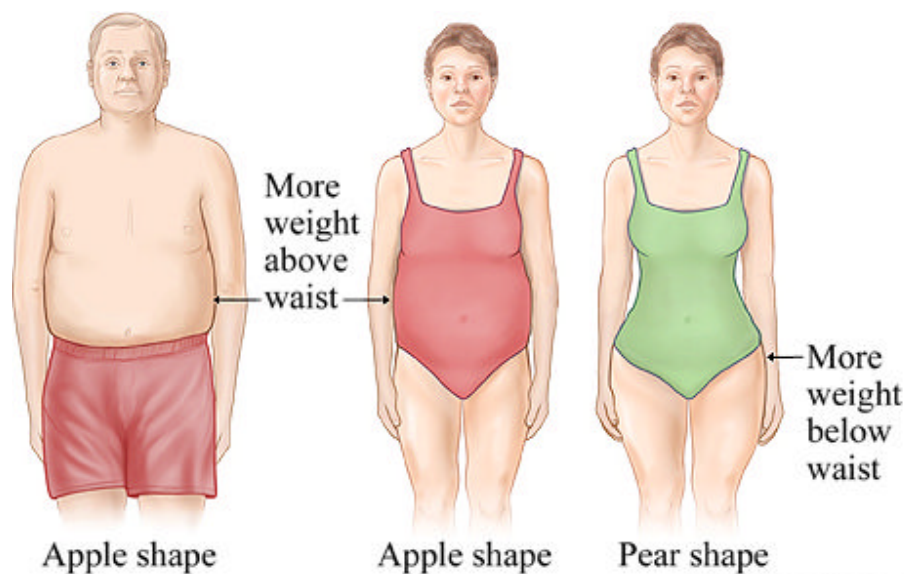


Figure 1.5.2.1 The Gender differences in fat distribution, male pattern android ‘apple’ and women ‘pear shaped’, that alters with age (menopause) and weight gain to more android shape in women. Adapted from book entitled ‘Medical Review: E. Gregory Thompson, MD - Internal Medicine’

Studies have shown that adipokines such as leptin (Van Harmelen *et al*, 1998) are secreted more abundantly in subcutaneous adipose depots, whereas adiponectin, IL1 β , IL8 and PAI-1 are more abundant in visceral adipose tissue (Motoshima *et al*, 2002). Adipokines have an important and influential effect on metabolism.

1.5.3 Adipokines

As mentioned earlier, adipose tissue had been regarded as a storage depot but this belief has changed in 1994 with the discovery of leptin. Since then our understanding of adipose tissue has changed and a new uncharted territory of research has been unravelled (Wozniak *et al*, 2009). Leptin is a chemical mediator of hormone-like properties synthesised and secreted into the bloodstream by adipose tissue. Leptin and all the subsequently discovered chemical mediators were termed adipokines. This discovery of adipokines called for a new definition of adipose tissue. Adipose tissue has been redefined as an endocrine organ and is considered to be the largest endocrine organ in the human body because of its vast capacity to increase its mass and spread within the human body. Some adipokines are secreted by white tissue adipocytes such as leptin, adiponectin and Retinol Binding Protein-4 (RBP-4); while some adipokines are secreted by other cells within the adipose tissue from the stromal vascular fraction cells. Tumour Necrosis factor α (TNF- α), interleukin-6 (IL-6) and resistin are just examples of adipokines secreted by stromal vascular cells. However, some adipokines are secreted by both cell types.

Currently, the number of discovered adipokines exceeds over one hundred. Research thus far has shown that adipokines influence and play roles in energy homeostasis, metabolism and inflammation (Gnaci ska *et al*, 2009). Adipokines provide an explanation for morbidities caused and associated with obesity and are the link by which adipose tissue influence and interact with other body organs. Due to the wide range of secreted adipokines and their broad array of physiological effects, adipokines have been classified and labelled according to their physiological effects.

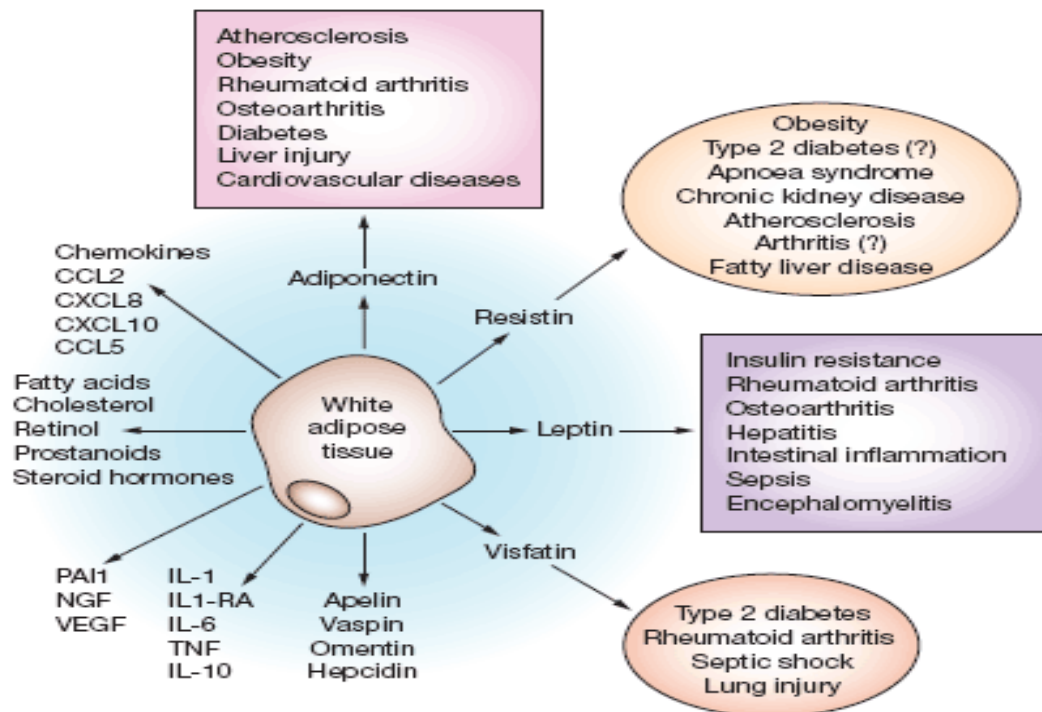


Figure 1.5.3.1 The multiple roles of Adipokines (Adapted from Lago *et al*, 1007).

Most notably, adipokines have been classified according to their hormonal effects on metabolism or their inflammatory effects (**Figure 1.5.3.1**) (Gnaci ska *et al*, 2009; Wozniak *et al*, 2009; Balistreri *et al*, 2010). It should be noted that those two effects are not mutually exclusive of each, thus creating a grey area of interaction between them. Adipokines exert an autocrine and paracrine effects as well as systemic. The sheer number of adipokines and their various physiological effects coupled with the fact that secretion of some adipokines is tissue site-specific mirror the complexity of adipose tissue (**Figure 1.5.3.2**) (Wozniak *et al*, 2009). Nonetheless, the jigsaw is yet far from being assembled. Examples of secreted adipokines are

shown in the following figure and are categorised according to their physiological effects. Some adipokines will be explored in more detail as these are examined within the main body of the thesis.

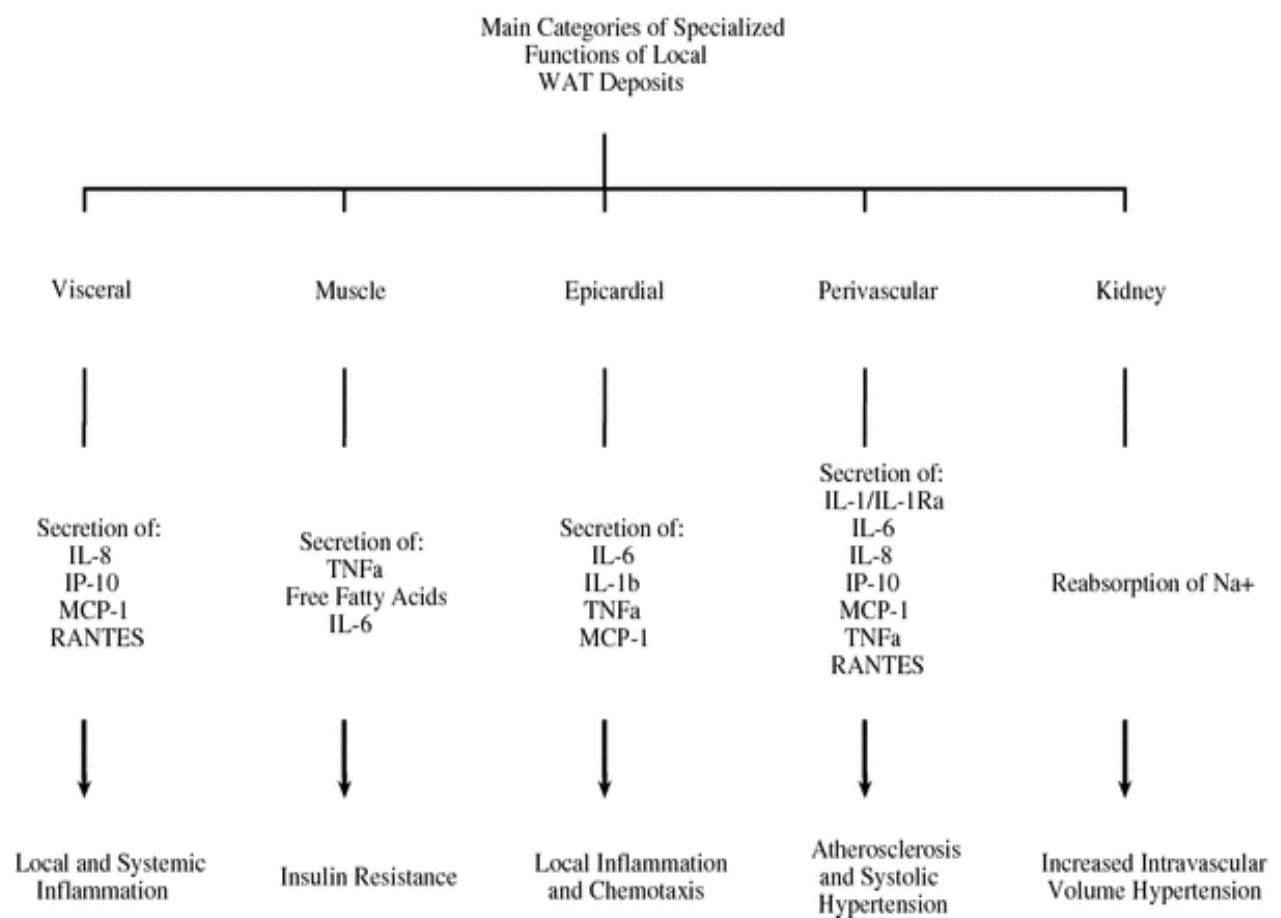


Figure 1.5.3.2 This figure illustrates site-specific adipokines expression in white adipose tissue including their local and system physiological effects. Adapted from (Wozniak *et al*, 2009).

1.5.4 Metabolic Stress

Obesity has been characterised as a morbidity of epidemic proportions which predisposes as well as coexists with other diseases such as T2DM, CVD and inflammation. So the question that follows is how does obesity prelude to those pathologies?

At this point it is worth pausing to sum up what goes on during obesity. What follows are the main event that categorise the state of obesity.

1. Excess of nutrients or positive energy balance
2. Adipocytes become hypertrophied
3. Which leads to hypoxia within hypertrophied “obese” adipose tissue and production of ROS
4. Secretion of pro-inflammatory adipokines from adipose tissue increases
5. Existence of hypertriglyceridemia and ectopic adiposity
6. Both of points number 4 and 5 result in tissue insulin insensitivity and ultimately insulin resistance and T2DM.

Secretion of pro-inflammatory adipokines and ectopic adiposity show an increase in adipose tissue activity which has negative implications (Figure 1.5.4.1). Although adipocytes have a high threshold for accumulating fat; it is that excess fat accumulation which triggers adipocytes to produce pro-inflammatory adipokines.

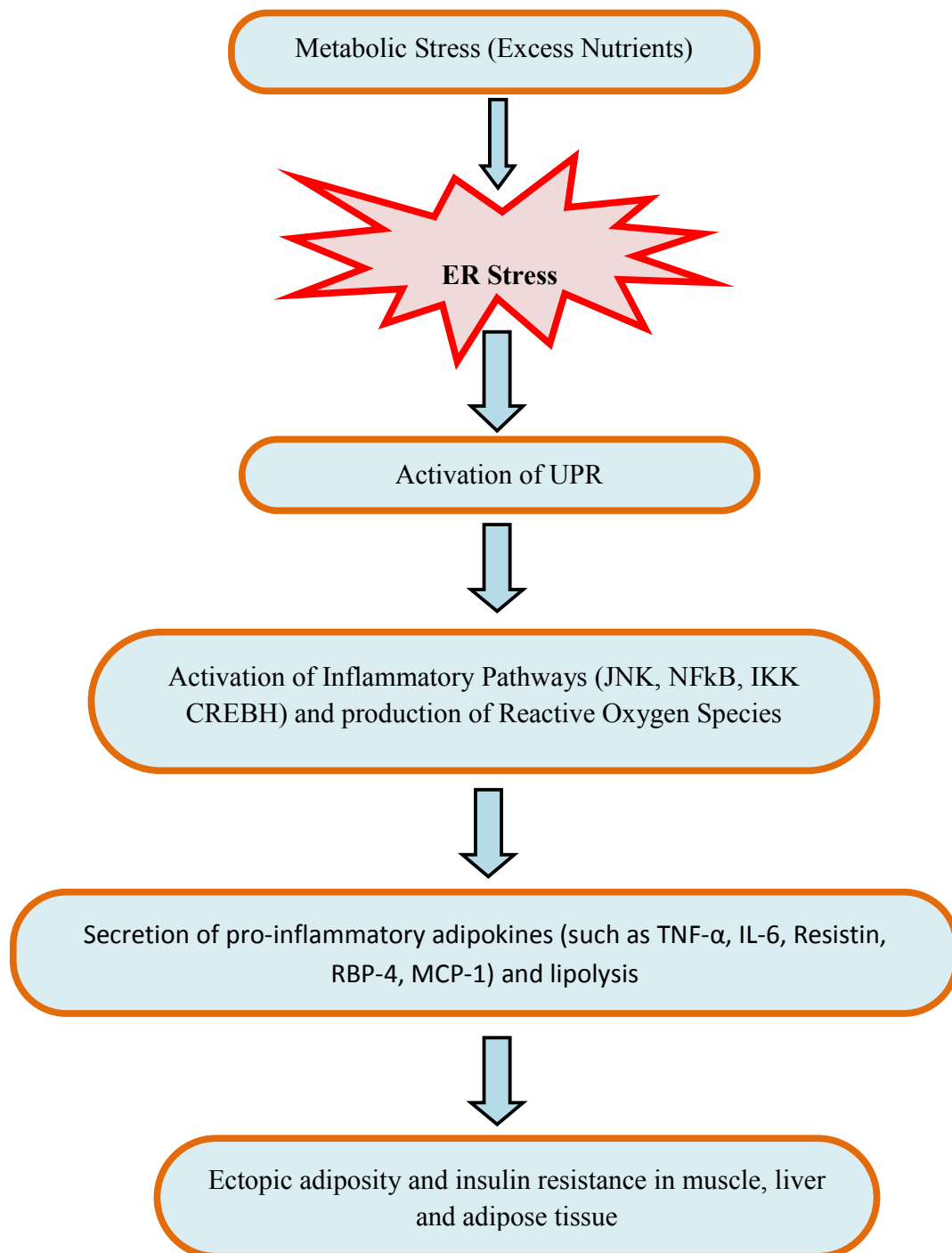


Figure 1.5.4.1 This figure highlights the key events leading to secretion of inflammatory adipokines due to obesity and ER stress

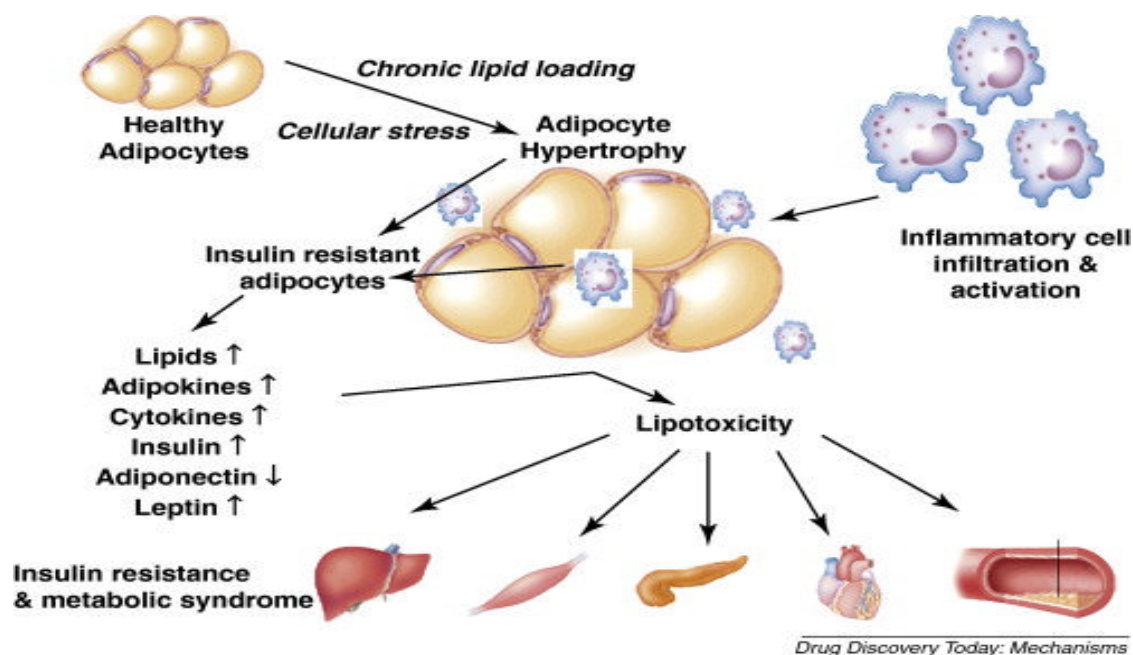


Figure 1.5.4.2 This figure shows interaction between adipose tissue and other tissues. Adapted from (Iyer & Brown, 2011)

In the state of obesity, adipose tissue elicits an inflammatory response by producing high levels of pro-inflammatory adipokines such as $\text{TNF-}\alpha$, IL-6, MCP-1 and others. Those pro-inflammatory adipokines act as a chemo attractant to circulatory immune cells such as macrophages (Sell & Eckel, 2010; Suganami & Ogawa, 2010). Therefore in obese subjects, adipose tissue becomes infiltrated with an additional number of macrophages and adipose tissue becomes inflamed (Figure 1.5.4.2). Thus from pathological point of view, obesity is classified as a chronic state of inflammation. Hyperlipidemia and ectopic adiposity contribute to the spread of hypertrophied adipose tissue depots within the viscera which ultimately exacerbate the inflammatory effects of obesity. Obesity can exist in a person for many years and as such a slow

low level mediator of inflammation can arise for many years leading to stress of cellular mechanisms (Wellen & Hotamisligil, 2005; Day, 2006; Nishimura *et al*, 2009); the mechanisms by which stress is initiated within the ER will comprise considerable focus of this current thesis.

Adipose tissue can accommodate large quantities of triglycerides in form of fat droplets. The common route to accumulate fat is by hyperplasia (increases in adipocytes number). However, if there is high consumption of nutrients then the positive balance of energy stored as fat and this creates an overload on adipocytes. In order to cope with increasing demand for fat storage, adipocytes become hypertrophied and the rate of hypertrophy exceeds that of hyperplasia (de Ferranti & Mozaffarian, 2008). Hypertrophied adipocytes become stressed if the energy balance is not restored. Cellular stress caused by excess nutrients is referred to as metabolic stress which adversely affects sub cellular organelles of metabolic functions such as endoplasmic reticulum (ER) and mitochondria as illustrated in figure 1.5.4.3 (de Ferranti & Mozaffarian, 2008; Gregor & Hotamisligil, 2007; Hotamisligil, 2010). Endoplasmic reticulum is involved in protein synthesis and folding, lipid synthesis and regulation of calcium storage; whereas mitochondria are involved in cellular respiration and production of energy.

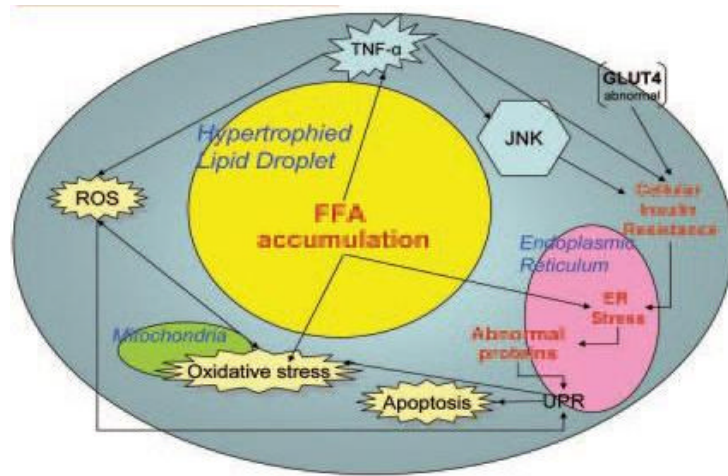


Figure 1.5.4.3 The figure shows hypertrophied adipocytes. Adapted from (de Ferranti & Mozaffarian, 2008)

1.6 Endoplasmic Reticulum (ER)

ER is a membranous structure in the form of completely enclosed convoluted tubular sac. It surrounds the nucleus whose membrane is continuous with that of the ER. The endoplasmic reticulum exists in two forms, rough and smooth. The rough ER has ribosomes attached to it during protein synthesis, whereas the smooth ER is free from ribosomes.

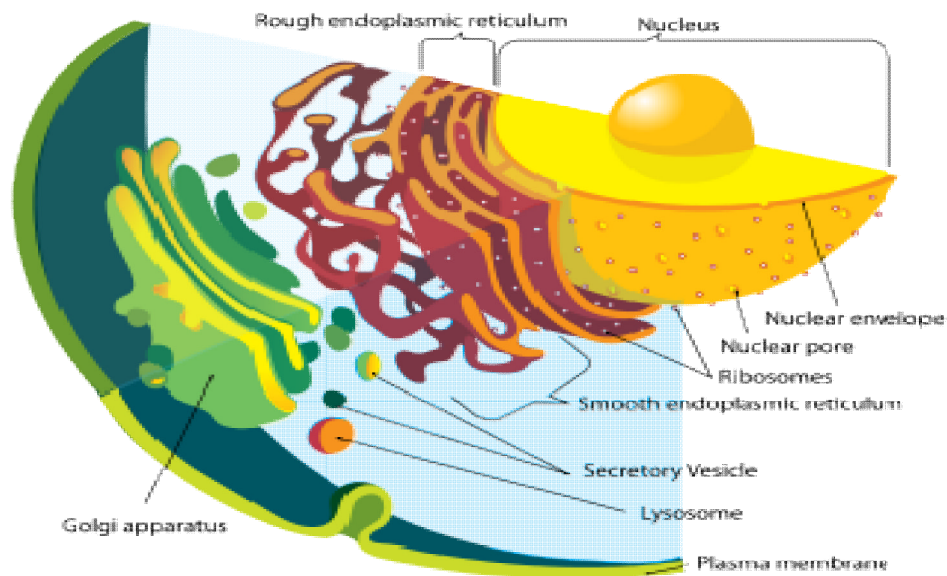


Figure 1.6.1 Schematic diagram of Endoplasmic Reticulum and Golgi Apparatus. Reproduced from www.vcharkarn.com

The endoplasmic reticulum has the following functions:-

1. Synthesis of plasma membrane and secreted proteins
2. Proper folding of those proteins
3. Synthesis of lipids and sterols
4. Storage of free calcium

It is self evident that the proper functionality of ER is vital for any cell to sustain life and all of the ER functions are pivotal in this respect. Nonetheless, ER can malfunction if it is exposed to either physiological or pathological insults. ER malfunction is commonly called ER stress;

whereby physiological stress occurs when there is an increased demand for protein production and pathological stress occurs as a result of accumulation of misfolded proteins or viral infection. ER stress can be due to genetic mutation or hereditary genotype (Lin *et al*, 2008; Yoshida, 2007).

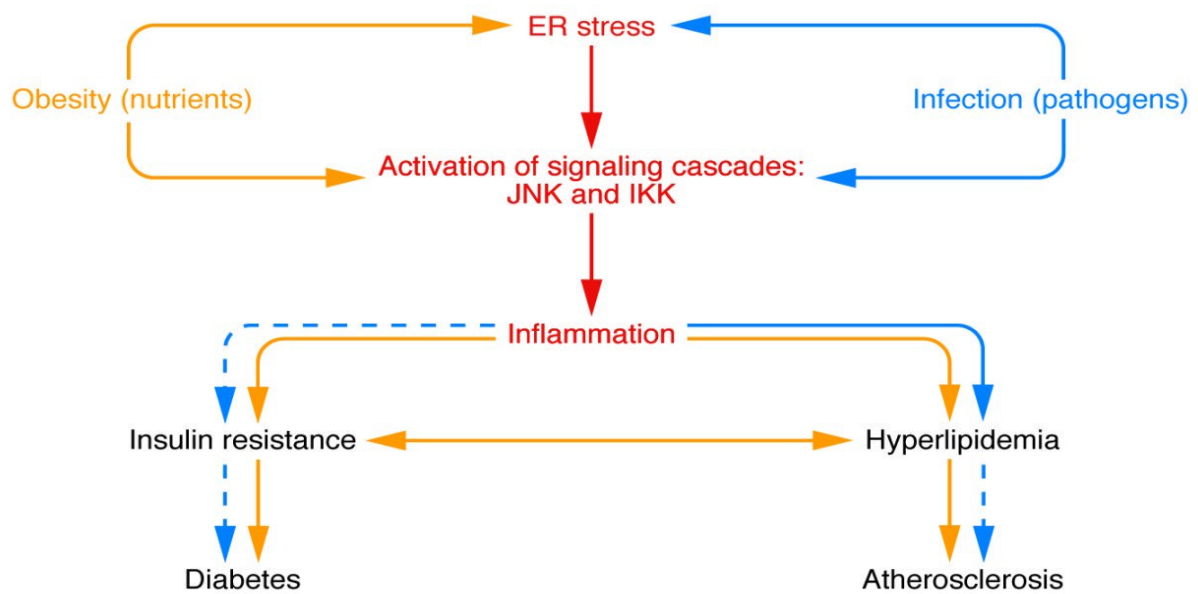


Figure 1.6.2 This diagram shows stressors of ER and resultant pathologies. Adapted from (Wellen & Hotamisligil, 2005)

Due to the highly critical and important functions carried by the ER, its malfunction is associated with a wide range of diseases as shown in following table.

Disease	Role of ER stress
Alzheimer's disease (AD)	<ul style="list-style-type: none"> ● Exact implications of ER stress in AD is unclear (AD brains show increase of protective UPR proteins) ● AD-associated mutant Presenilin 1 induces ER stress response with suppression of protective UPR signalling
Parkinson's disease (PD)	<ul style="list-style-type: none"> ● Unclear whether ER stress in PD is mainly protective or contributory to disease ● Parkin suppresses ER-stress-induced cell death ● Parkin expression is controlled by ER stress ● Parkin mutants associated with PD
Amyotrophic lateral sclerosis	<ul style="list-style-type: none"> ● Mutant SOD interferes with ER-assisted degradation machinery and activates ASK1
Polyglutamine disease	<ul style="list-style-type: none"> ● Polyglutamine induces the UPR and suppresses proteasomal activity
Prion disease	<ul style="list-style-type: none"> ● Brains affected with prions show induction of ER chaperones, implying protective UPR against ER stress
Stroke	<ul style="list-style-type: none"> ● Ischaemia induces ER stress in neurons, activates the UPR and finally leads to neuronal apoptosis associated with CHOP induction and ASK1 activation
Bipolar disease	<ul style="list-style-type: none"> ● Medications for treating bipolar disease induce the UPR
Heart disease	<ul style="list-style-type: none"> ● Induction of ER stress by ischaemia in the heart leads to degeneration of cardiac myocytes ● Transaortic constriction induces expression of ER stress ● Myocardial infarction induces the UPR
Atherosclerosis	<ul style="list-style-type: none"> ● Oxidized lipids and homocysteins induce ER stress in vascular cells, cholesterol in macrophages
Type 1 diabetes	<ul style="list-style-type: none"> ● Impaired PERK pathway is responsible for type 1 diabetes (Wolcott-Rallison syndrome)
Type 2 diabetes	<ul style="list-style-type: none"> ● Obesity (a cause of type 2 diabetes) induces ER stress, leading to insulin resistance
Type 2 diabetes	<ul style="list-style-type: none"> ● Fatty acids (palmitate) induce apoptosis of β cells
Cancer	<ul style="list-style-type: none"> ● Protective UPR proteins are upregulated in cancer cells subjected to hypoxic environments
Autoimmune disease	<ul style="list-style-type: none"> ● ER protein overload may contribute to autoantigen production ● GRP78 can be an autoantigen

Table 1.6.1 List of ER stress-related diseases. Adapted from (Kim *et al*, 2008)

Table 1.6.1 illustrates that stressed ER in different tissues lead to different diseases. Thus the importance of ER cannot be over emphasised. To understand the mechanisms of dysregulation in the ER during stress, it is worth while reviewing the normal function of ER. Steps of protein synthesis and folding are outlined below:-

1. Protein synthesis is initiated when mRNA strand attaches to the ribosome for translation
2. This attachment translocates the ribosome to the ER membrane surface to begin protein translation in a process called cotranslation.
3. Translocation and attachment of the ribosome-mRNA unit to surface causes ER membrane to open at the point of attachment (called translocon) and translation begins
4. The synthesised chain of amino acids enters the ER lumen as it is synthesised.
5. Once translation process is complete, translocon closes and the ribosome-mRNA unit dissociates from the ER membrane surface
6. The newly synthesised amino acid chain is processed and folded into its correct conformation. Processing and folding of the amino acid chain is handled by special ER proteins called chaperone proteins. Processing of the newly synthesised amino acid chain entails addition of a carbohydrate entity (as is the case of glycosylated proteins), proper positioning of a sulphide bond (carried out by PDI) or imposing a specific allosteric conformation (either cis or trans) which is performed by peptidyl proline isomerase (PPI).
7. If correct folding of the newly synthesised protein cannot be attained due to genetic mutation for example, this misfolded or unfolded protein is retrotranslocated to the

cytosol for degradation by the proteasomes. This process is referred to as ER Assisted Degredation (ERAD).

8. Upon completion of ER-assisted folding, the newly synthesised protein is exported to the Golgi apparatus for further modification, if required, and finally the fully mature protein is exported from Golgi to either become an integral part of the plasma membrane or be secreted by the cell.

As mentioned in step 6 of the outline, protein processing and folding are handled by the ER luminal proteins called chaperones. As such any malfunction of those proteins leads to improper processing and eventually misfolded final protein product. ER has the ability to sense any improper processing or misfolding within the ER lumen, so that any processing error may be rectified. In a state of obesity, adipocytes are bombarded with a continuous high level of lipids which leads to hypertrophy. Hypertrophy exerts an extra pressure on the metabolic machinery of adipocytes in order to keep up with the increasing demand, which in turn is translated into ER stress and oxidative stress in the mitochondria. ER stress can accommodate an increase in metabolic demand up to the chaperones capacity to handle newly synthesised proteins. If this capacity is exceeded, then misfolded proteins start to accumulate within the lumen of the ER. This accumulation calls on the activation of a rescue response termed Unfolded Protein Response (UPR) (Gregor & Hotamisligil, 2007; Boden *et al*, 2008; Karalis *et al*, 2009; Hotamisligil, 2010; Hummasti & Hotamisligil, 2010).

1.6.1 Unfolded Protein Response (UPR)

The unfolded protein response (UPR) team consists of a group of ER protein who work closely together. Those proteins act as molecular sensors and regulators of the UPR. The UPR main aim is to relieve ER from its overload or stress. Notwithstanding if the relief efforts fail then UPR triggers apoptic pathway (Kim *et al*, 2008; Xu *et al*, 2005). The UPR exerts its action by recruitment of the proteins which in turn activate the three different pathways. The ultimate result of those pathways is to increase production of chaperone proteins in order to cope with the increasing demand for protein synthesis, however if the demand still exceeds ER capacity to recover, then cell death is the other resort. As illustrated in the figure 1.6.1.1, the three main proteins of UPR are inositol-requiring protein-1(IRE- α), activating transcription factor-6 (ATF-6) and protein kinase-like ER kinase (PERK). Those proteins span the ER membrane with their N-terminus in the lumen of the ER and their C-terminus in the cytosol. Normally, those proteins are inactivated by the association of a sensory protein known as Binding immunoglobulin Protein (BiP). ER stress results in BiP dissociation from IRE- α , ATF-6 and PERK which in turn activates the UPR proteins. The three active forms of UPR proteins initiate a cascade of events leading to

1. Attenuation of global mRNA translation so the influx of proteins into the ER is halted temporary
2. An increase in the transcription of chaperone proteins to facilitate the existing increasing demand for protein folding
3. An increase in the induction of the ERAD proteins
4. Finally if all fails, pro-apoptotic pathways are activated

Many studies propose that ER stress is one potential route which links obesity to insulin resistance and T2DM. This study has ER stress as a focal point of investigation with the UPR as a whole and its constituent components are used as an explorative tool to examine the ER stress in obesity linkage to insulin resistance and T2DM. As stated earlier, UPR exerts its adaptive effects via three arms of action as shown in the figure 1.6.1.1.

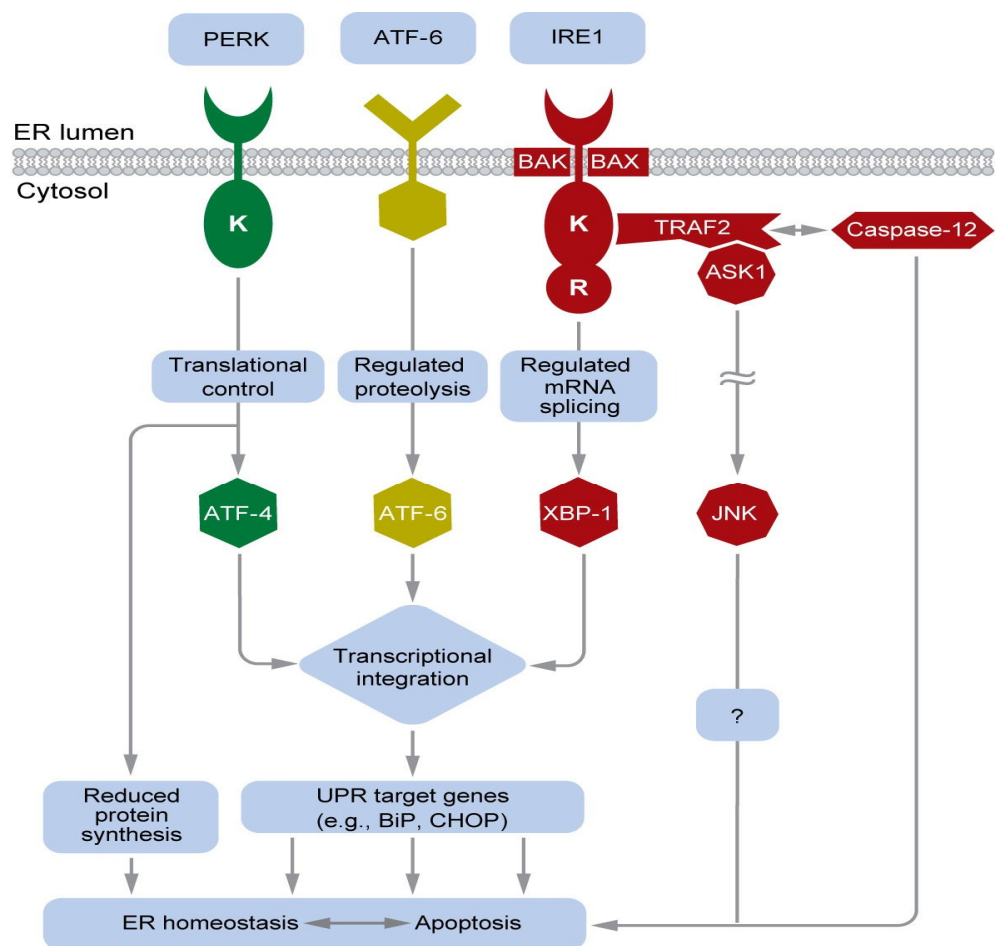


Figure 1.6.1.1 Pathways of Unfolded Protein Response (Adapted from Lin *et al*, 2008)

1.6.1.1 IRE-1 α Pathway

The IRE-1 α UPR protein is a transmembrane protein weighs approximately 100 kDa and has two domains; serine/threonine kinase domain and an endoribonuclease domain. The dissociation of the ER sensory protein BiP causes IRE-1 α to oligomerise. This oligomerisation activates IRE-1 α mRNAase domain which is located in the cytosolic side of the ER membrane. IRE-1 α mRNAase splice the mRNA of XBP-1 protein (Kim *et al*, 2008; Hummasti & Hotamisligil, 2010; Ron & Walter, 2007; Yoshida, 2007; Marciniak & Ron, 2006). This spliced mRNA is translated and the resulting protein translocates to the nucleus to induce an increased rate of expression of

- i) ER chaperone proteins
- ii) ERAD proteins
- iii) Lipid synthesis proteins and
- iv) ER biogenesis proteins

1.6.1.2 ATF-6 Pathway

The second arm of the UPR is the ATF-6 protein. In response to ER stress, ATF-6 is released from BiP and is translocated to the Golgi apparatus for cleavage by resident proteins S1P (Site 1 Protease) and S2P (Site 2 Protease). The cleaved ATF-6, also called ATF-6f (“f” for fragment) is released into the cytosol to enter the nucleus and regulate gene expression. ATF-6 pathway activation results in the upregulation of the ER chaperone proteins (Marciniak & Ron, 2006; Yoshida, 2007; Ron & Walter, 2007; Fong *et al*, 2004; Kim *et al*, 2008).

1.6.1.3 PERK Pathway

PERK mechanism of activation is similar to that of IRE-1 α as both possess a serine/threonine kinase domain protruding into the cytoplasm. Once BiP dissociates, PERK is activated by oligomerisation and autophosphorylation. This active form of PERK causes the phosphorylation of the α -subunit of eukaryotic translational Initiation Factor 2 (eIF2 α) protein in the cytoplasm. This phosphorylation inactivates eIF2 α which results in attenuation of global proteins translation. Thus decreasing the protein folding load on the ER chaperon proteins (Kim *et al*, 2008; Bastard *et al*, 2006; Ron & Walter, 2007; Hummasti & Hotamisligil, 2010; Marciniak & Ron, 2006; Yoshida, 2007).

1.6.1.4 Pro-apoptotic pathway

The aim of the UPR is to relieve the ER from its overload and stress firstly by increasing the expression of the ER chaperone and biogenesis proteins in addition to attenuating the translation of global proteins. If the ER functional integrity cannot be restored through those adaptive processes, then apoptosis is induced as a last resort option. Basically, each of the three UPR pathways plays a role in initiating the apoptotic process albeit some are more prominent than others. The most prominent and direct route is triggered through PERK pathway. Although the activation of PERK has an inhibitory effect on eIF2 α which subsequently attenuates global protein translation, interestingly, inactivated eIF2 α results in increased translation of ATF-4 (Activating Transcription Factor-4), whose target gene is C/EBP homology protein (CHOP). CHOP is a protein involved in inducing apoptosis and its gene promoter has binding sites for ATF-6 α as well as XBP-1s. So that ATF-6 and IRE-1 α can trigger apoptosis via this route. However, IRE-1 α has a distinct apoptosis-inducing pathway. Through its protein kinase activity, the cytoplasmic portion of IRE-1 α binds to TRAF2 (Tumour necrosis factor Receptor-Associated Factor 2), an adaptor protein forming a unit which then binds to and forms a complex with ASK1 (Apoptosis Signal-regulating Kinase 1). This complex, IRE-1 α – TRAF2 – ASK1, phosphorylates JNK (Jun Kinase) protein has a pro-apoptotic effect (Yoshida, 2007; Marciniak & Ron, 2006; Hummasti & Hotamisligil, 2010; Kim *et al*, 2008; Ron & Walter, 2007).

1.7 ER Stress and Insulin Resistance

Some studies have noted that ER stress is associated with insulin insensitivity and ultimately to insulin resistance (Ozcan *et al*, 2006; Hummasti & Hotamisligil, 2010; Hotamisligil, 2010; Karalis *et al*, 2009; Gregor & Hotamisligil, 2007; Boden *et al*, 2008; Nakatani *et al*, 2005). One route could be the activation of JNK/IKK which causes inhibition of IRS-1 and IRS-2. Activation of JNK/IKK leads to phosphorylation of IRS-1 and IRS-2 at the serine residue, thus rendering the plasma insulin receptor non-responsive to circulating insulin.

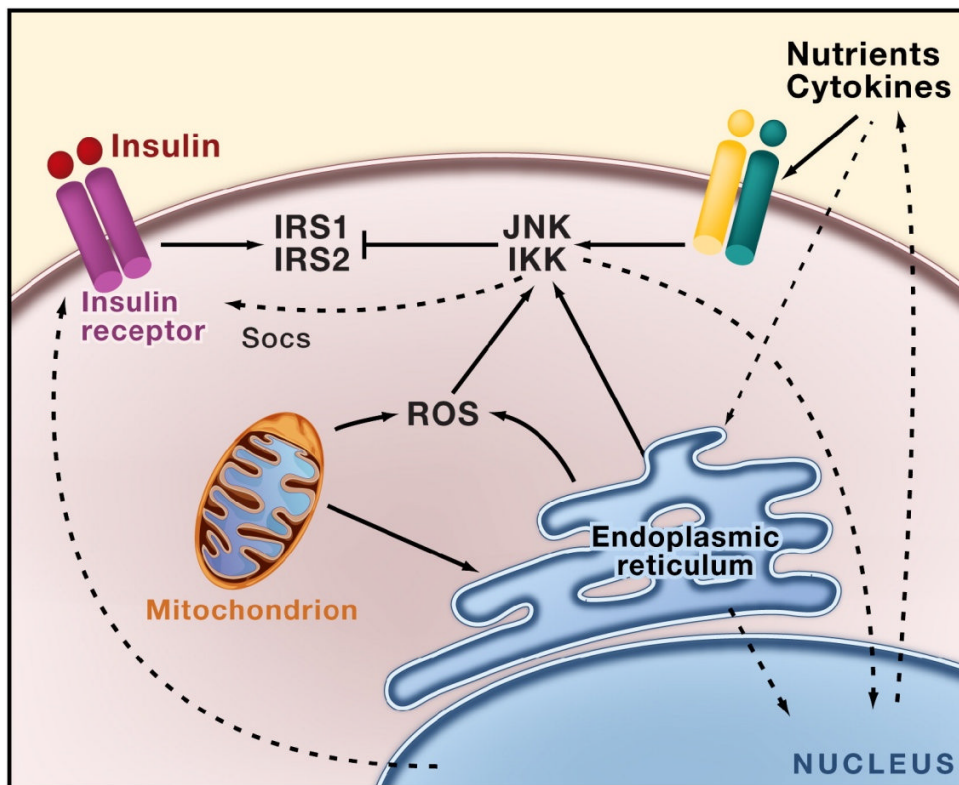


Figure 1.7.1 Cross talk between ER, inflammatory cytokines and Insulin Receptor. Adapted from (Hotamisligil, 2010)

1.8 ER Stress and Inflammation

Similarly, ER stress pathways intersect with inflammatory pathway at JNK/IKK (Figure 1.8.1). It is well documented that JNK/IKK is one of the main pathways of inflammation (Jiao *et al*, 2010; Hotamisligil, 2008; Karalis *et al*, 2009; Hotamisligil, 2010; Hummasti & Hotamisligil, 2010). The trigger of this pathway leads to production of pro-inflammatory adipokines. This may create an additional burden on ER internally and externally. Internally, ER has to cope with a further increasing demand for pro-inflammatory adipokines synthesis. Externally, if those pro-inflammatory adipokines were to be successfully synthesised and secreted, they would exert their pro-inflammatory effects on an autocrine and paracrine level, thus creating an even worse vicious cycle as depicted in figure 1.8.2.

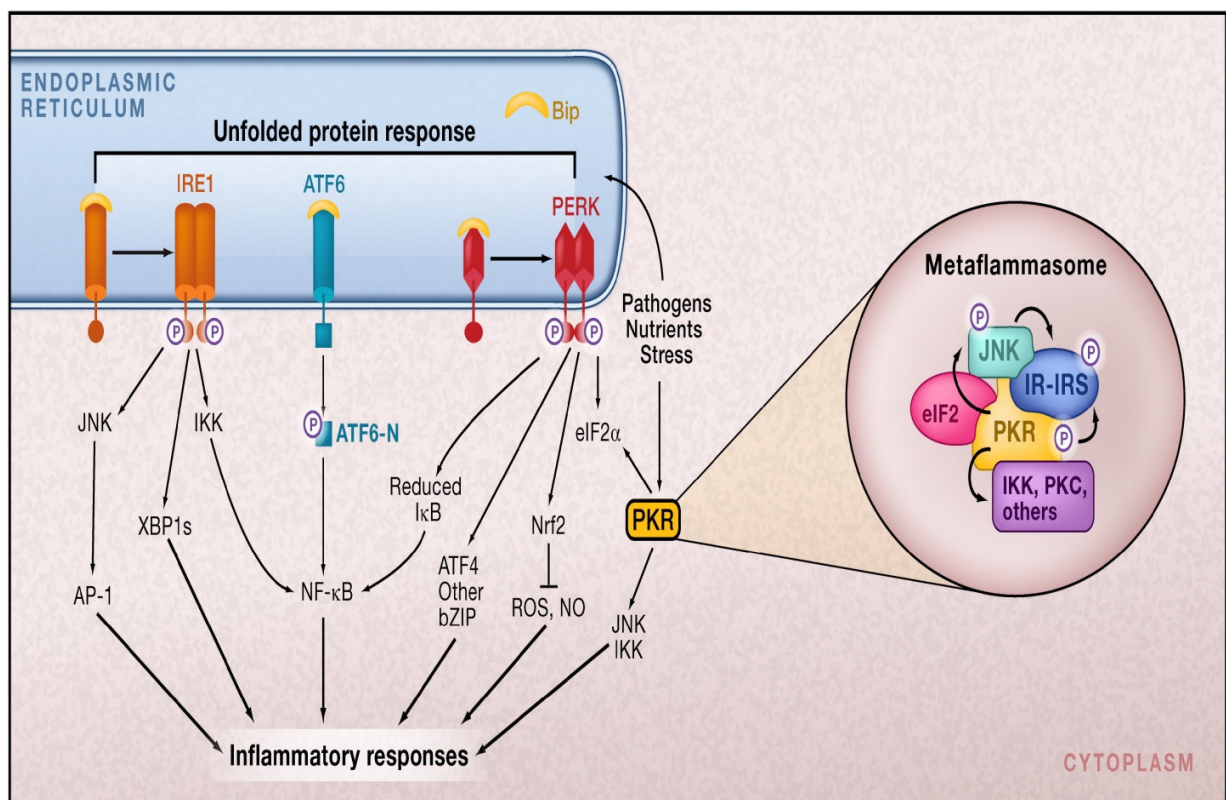


Figure 1.8.1 ER stress and Inflammation. Adapted from (Hotamisligil, 2010)

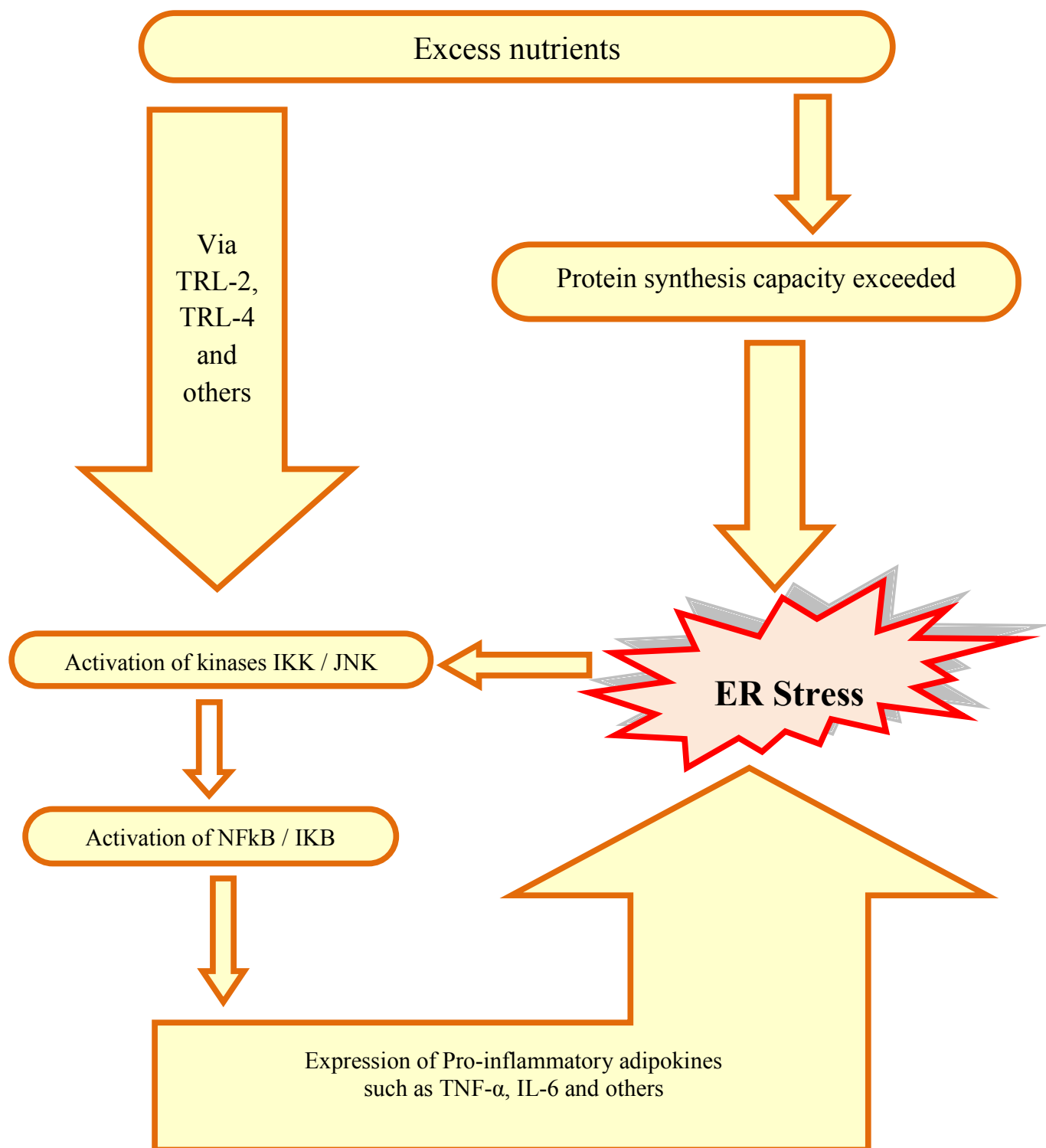


Figure 1.8.2 This figure shows the cascade of events leading to secretion of inflammatory adipokines thus creating a vicious cycle of ER stress.

1.9 Anti-inflammatory Chemicals

Due to the close link between ER stress and inflammation represented by JNK/IKK activation, chemicals of anti-inflammatory properties have been used experimentally to relief ER stress. The most common and widely used anti inflammatory drug is salicylate which is commercially known as ASPIRIN. Salicylate exerts its anti-inflammatory action by blocking the activation of NF κ B, thus interrupting the inflammatory pathway and the production of pro-inflammatory adipocytokines (Yuan *et al*, 2001; Amann & Peskar, 2002; Yin *et al*, 1998; Kopp & Ghosh, 1994).

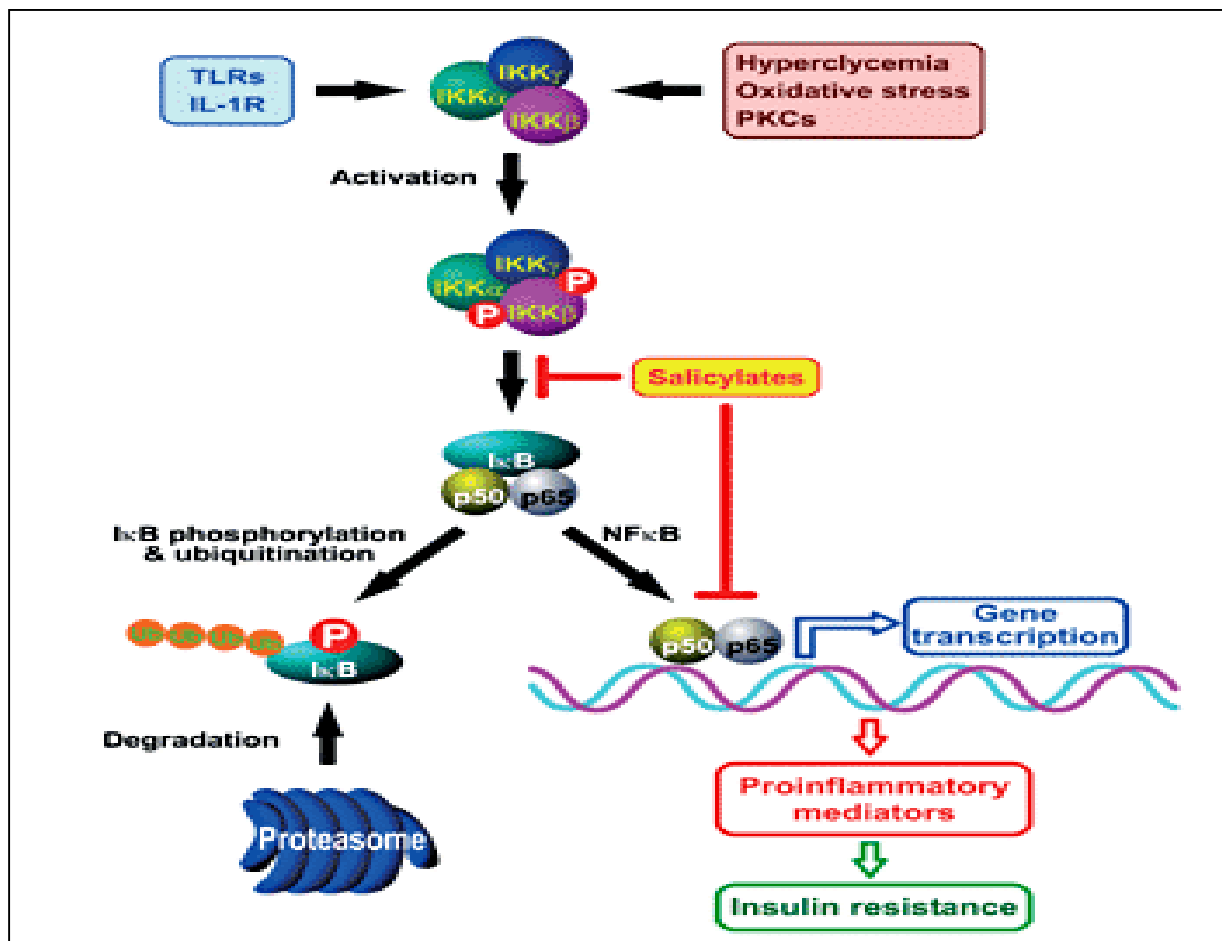


Figure 1.9.1 This figure shows salicylates mode of action. Adapted from (Davies *et al*, 2007)

1.10 Aims of Study

The study was designed to explore specific points related to ER stress in human adipose tissue. Cultured differentiated human pre-adipocytes were used as a model to mimic certain pathologies that result from or co-exist with obesity. The aims of the study can be summarised in the following points:-

1. To characterize cultured differentiated human pre-adipocytes as a valid model for human adipose tissue.
2. To examine the ER stress marker proteins in the adipose tissue of lean and obese subjects.
3. To investigate the origins of ER stress in primary human adipocytes in metabolic stress states of hyperglycemia, hyperlipidemia and endotoxicity.
4. To examine the effect of salicylate as an anti inflammatory agent on ER stress.

Taken together this thesis sought to explore the potential mediators of sub-clinical inflammation and the effect such stimulants have on ER stress and whether an anti-inflammatory agent could abate such ER stress and what pathways are affected.

Chapter 2

General Methods & Materials

The following chart summarizes the main laboratory procedures discussed in this chapter.

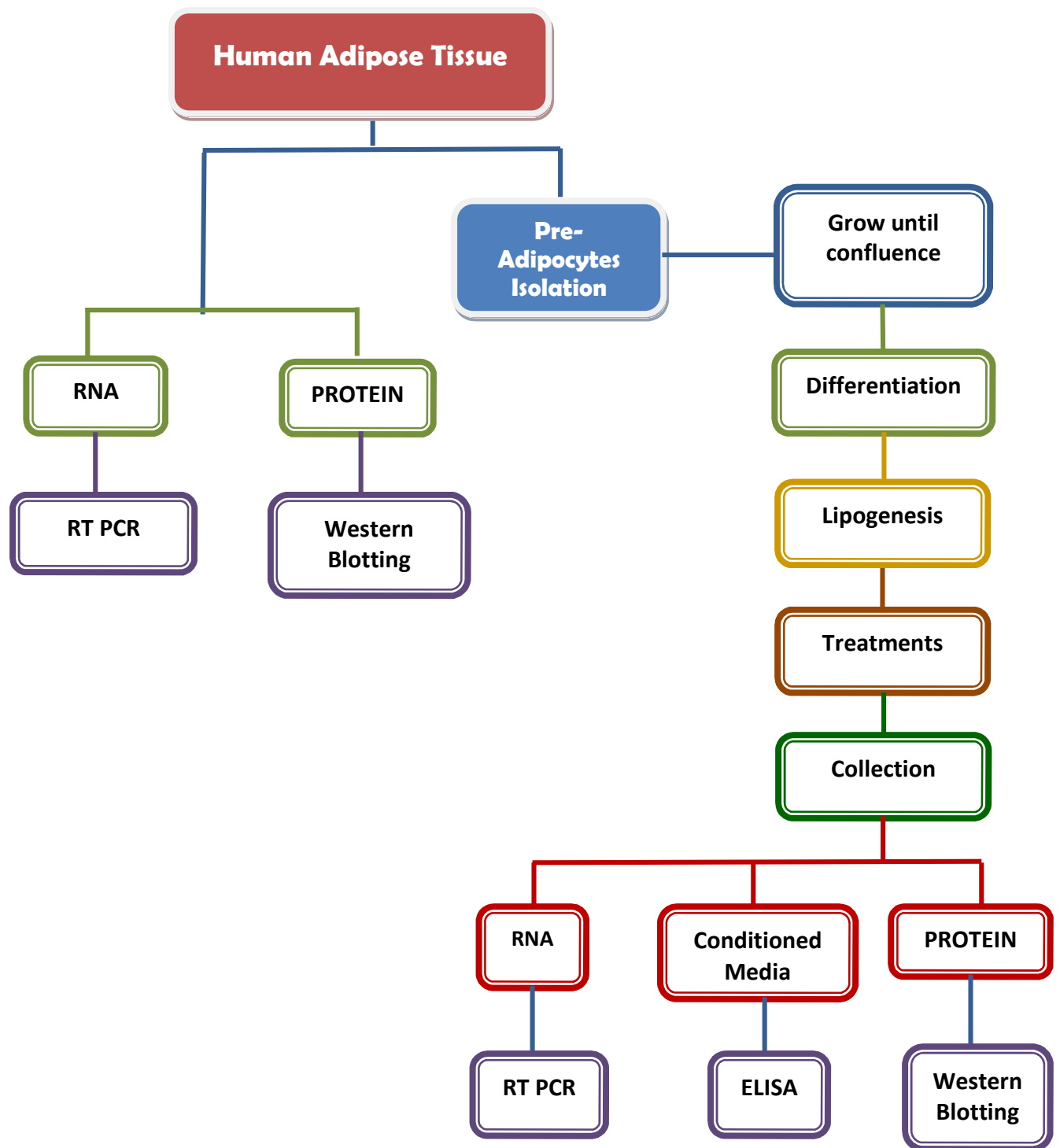


Figure 2.1 A chart of laboratory procedures followed during the course of this study

This chapter details the methods and materials most frequently used throughout the thesis. Precise information outlining specific methods for each study will be covered within each appropriate chapter. Information on recipes of all in-house made reagents is mentioned in the Appendices as well as a list of manufacturers and companies used to purchase reagents.

The starting cellular materials were human adipose tissue, preadipocytes extracted from the human adipose tissue.

Abdominal Subcutaneous (AbSc) adipose tissue was collected from subjects undergoing elective cosmetic surgery (liposuction) in accordance with local ethics committee guidelines. All subjects with a history of malignancies or under treatment or suffering from any endocrine abnormalities were excluded from the study. The fresh primary adipose tissue was utilised in the following ways; either frozen and stored or processed to isolate preadipocyte cells.

2.1 Freezing Adipose Tissue

Approximately 40 ml of adipose tissue obtained from liposuction was aliquoted into sterile 50 ml centrifuge tubes then flash frozen in liquid nitrogen. To ensure minimal protein damage or degradation, frozen AbSc adipose tissue samples were placed directly from liquid nitrogen into a -80°C freezer for storage until further use.

2.2 Isolation of Preadipocyte Cells: Collagenase Digestion

Adipose tissue (25 ml) obtained from liposuction was poured directly into sterile 50 ml centrifuge tubes. In order to aid the mechanical break-up of adipose tissue into its different cellular components, the enzyme collagenase was used. Adipose tissue was suspended in 20 ml of warm collagenase solution then incubated at 37°C in a

continuous shaking (100 cycles/min) water bath for 30-45 min, with additional shaking at 10 min intervals until the consistency of the adipose tissue was a smooth homogeneous liquid. Following collagenase digestion, adipose tissue was filtered through a sterile fine cotton mesh into another sterile 50 ml centrifuge tube. Centrifugation at 360 x g for 5 min separated the collagenase digested adipose tissue into three independent layers: the lower stromal-vascular fraction containing a pellet of pre-adipocyte cells, the central adipocyte layer and the upper supernatant fraction containing lysed adipocyte cells. The supernatant fraction and the adipocyte layer were discarded whereas the pellet was resuspended in lysis buffer and left at room temperature for 20 minutes. The suspension was centrifuged at 2000 rpm for 5 minutes. The resulting supernatant was discarded and the pellet was resuspended in a mixture of DMSO and foetal calf serum. The suspension was aliquoted in cryogenic vials and stored at -80 C overnight then in liquid nitrogen until further use.

2.3 Extraction of Protein from Whole AbSc Adipose Tissue using RIPA

Protein samples from whole AbSc adipose tissue to be analysed by Western blotting were extracted using RIPA buffer. Whole adipose tissue was collected from -80°C storage, then, without allowing the sample to defrost, ~100 mg of adipose tissue was cleaved off using a sterile scalpel and forceps then transferred into a 5 ml bijou tube. RIPA buffer (600 µl) was added to the tissue. The sample mixture was then homogenised using a rotor-stator homogeniser at 1100-4200 x g for 3-5 s. The homogenised mixture was then transferred to 1.5 ml microcentrifuge tubes and flash frozen in liquid nitrogen. Once thawed, the samples were centrifuged at 60,060 x g for 30 min at 4°C. Using a fine bore needle and syringe, liquid from below the solid layer

was extracted and aliquoted into fresh 0.5 ml microcentrifuge tubes and stored at -80 °C until further use.

2.4. Propagation and Differentiation of Extracted Primary Preadipocytes

Cryovials containing extracted primary preadipocytes were removed from liquid nitrogen and placed in a 37° C water bath for about 10 minutes. The thawed cells were plated in cell culture flasks containing growth culture medium. Growth culture medium consists of DMEM/F12 Ham phenol-free medium supplemented with glutamine (1%), foetal bovine serum (10%), transferrin (1%), penicillin (100 units/ml) and streptomycin (100 µg/ml). The growth medium was refreshed every 3 days until the cells reached about 80% confluence. At this stage, the cells were trypsinised, and plated in cell culture 6-well plates. Trypsinisation process involved aspirating the existing culture medium and washing the cells with phosphate buffered saline (PBS). PBS was then aspirated and trypsin was added to the cells to incubate for 5 minutes, after which, growth medium was added to inactivate trypsin. The mixture was mixed properly to ensure the detachment of all cells and uniformity of cellular distribution within the suspension. A small suspension (20 µl) was used to count the number of cells using a haemocytometer. This was to ensure that all wells had an approximately similar number of cells. Depending on the calculation result, equal volumes of cells-containing suspension was aliquoted in 6-well plates. Growth medium was refreshed every 3 days until the cells reached 100% confluence. Once confluent, preadipocytes differentiation was induced using differentiation medium (PromoCell, Germany). The cells were differentiated over 4 days with differentiation medium refreshed on day 3. On the fifth day, the medium was refreshed with nutrition medium (PromoCell, Germany) supplemented with penicillin and streptomycin. Nutrition medium was

refreshed every 3 days until lipid droplets formation; which was noted as an indication of differentiation. The process of lipid droplets formation within the cells was complete by 10 – 14 days.

At this stage, the cells were ready for the treatment experiments.

2.5 Experimental Treatment of Differentiated Primary Preadipocytes

In order to prepare the cells for the planned treatments, the cells underwent a wash-out period of all growth and biochemical factors. This procedure is referred to as Detoxification and is necessary to minimize any biochemical interference with any proposed treatments. Detoxification was carried out for 24 hours and involved changing the nutrition medium with detoxification medium, which consists of DMEM/F12 Ham and 3% foetal bovine serum only (Invitrogen, UK).

The detoxification medium was refreshed after 24 hours and the proposed treatments were added to each well as explained in the relevant results chapters. Cells cultured under different treatment regimes were extracted following the treatment period for protein and RNA analysis.

2.6 Protein and RNA Cellular Extraction

At the end of the specific experimental period, conditioned media were collected and aliquoted in eppendorfs and stored at -80° C to be analyzed by ELISA at a later stage. Cell culture wells containing differentiated preadipocytes were then washed twice with ice-cold PBS and gently tapped on the back of the plate against a tissue paper to remove any PBS excess. Plates were then placed on ice tray and 250 µl of Qiazol (Qiagen, Germany) was added to cells designated for RNA collection and 250 µl of ice-cold protein lysis buffer were added to cells designated for protein extraction.

Whilst waiting for the protein lysis buffer to incubate for at least 5 minutes, RNA collection was begun. The process of RNA collection comprised removing of cells manually with a scraper, aspirating the Qiazol-scrapped suspensions and pipetting it into eppendorfs tubes then flash freezing the tubes in liquid nitrogen. The tubes were then stored at -80° C.

Protein lysis buffer-containing cell culture wells were also manually scrapped and the suspensions were placed in eppendorf tubes and centrifuged in a pre-cooled centrifuge at a temperature of 4° C for 5 minutes at 16000 g. Once spun, a pellet and a supernatant were formed. The protein-containing supernatant is pipetted into new eppendorf tubes and stored at -80° C whilst the pellets were discarded.

2.7 Determination of Protein Concentration

Protein concentrations of samples from adipose tissue and differentiated primary adipocytes were measured according to the following procedures. To determine the protein concentration, Bio-Rad Protein Assay kit was used. Frozen aliquots of the extracted protein sample were thawed and vortexed to ensure a homogenous sample distribution. The Bio-Rad Protein assay kit constitutes a 5 fold concentrated dye reagent and a bovine serum albumin (BSA) protein standard in a lyophilized form.

The dye reagent was diluted with deionised water to yield 1X concentration. BSA was reconstituted with distilled water to yield a concentration of 1.4 mg/ml. Using the BSA reconstitute, seven protein standards were prepared of different concentrations (0, 1, 2.5, 5, 10, 15, 20 µg/ µl) where 0 µg/ µl was made from the dye reagent without the addition of any BSA and is regarded as a blank. Four micro litres of each extracted protein sample were added to 1 ml dye reagent and mixed in cuvettes. The sample mixtures were incubated for 30 minutes at room temperature. The absorbance of all

samples was measured against the blank at optical density of 595 nm using a spectrophotometer (6505 UV/VIS, Jenway, UK). The absorbance values of the seven BSA standards were used to plot the standard curve against which sample protein concentrations were read using their respective absorbance values.

2.8 Western Blot Analysis of Differentiated Preadipocytes

& Whole adipose Tissue

Western blotting is an immunoassay-based technique that allows the sizing, identification and quantification of specific proteins. In brief, proteins of interest undergo electrophoresis through a gel. Proteins are separated according to their molecular weight (M_r). Following electrophoresis, proteins are electrophoretically transferred onto a polyvinylidene-fluoride (PVDF)[™] or nitrocellulose membrane. Proteins of interest are then detected by probing with antibodies raised against them. Proteins are then visualised using a chemiluminescent detection system. This method is detailed below in full.

2.8.1 Preparation of Protein Samples for Electrophoresis

Quantified protein samples were thawed and vortexed. Equal volumes of protein were then transferred into 1.5 ml eppendorfs containing loading buffer, at a minimum ratio of 1:2 (sample: loading buffer) (**Table 2.8.1.1**). Varying quantities dH₂O were added to the sample mixture to standardise final volumes. Seven micro litres of a rainbow molecular weight marker (14,300-220,000 Da) was loaded alongside each set of samples (i.e. minimum 1 lane per gel), providing a visual protein size record with which protein samples could be compared. The sample mixtures were then heated at 95°C for 5 min to denaturise and linearise the proteins.

REAGENT	QUANTITY	FINAL CONCENTRATION
Tris HCl (pH 6.8)	625 μ l	125 mM
SDS (10%)	500 μ l	4%
Glycerol	1000 μ l	20% (w/v)
DTT	200 μ l	6.5×10^{-3} mM (w/v)
Bromophenol Blue	125 μ l	2.5×10^{-3} mM (w/v)
dH ₂ O	250 μ l	N/A

Table 2.8.1.1 Quantities of reagents in loading buffer for Western blot analysis.

2.8.2 Electrophoretic Protein Separation

SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was used to separate proteins according to their size (4% stacking gel, pH 6.8; 8-15% resolving gel, pH 8.8 (percentage of resolving gel was chosen according to the size of the protein of interest). The reagents and their quantities for each gel used throughout the thesis are shown in Tables 2.8.2.1 and 2.8.2.2.

RESOLVING GEL	QUANTITY FOR 8% GEL	QUANTITY FOR 12% GEL	QUANTITY FOR 15% GEL
REAGENT			
Protogel*	5.3 ml	8 ml	9.9 ml
Protogel Resolving Buffer*	5.2 ml	5.2 ml	5.2 ml
dH ₂ O	9.3 ml	6.6 ml	4.5 ml
Ammonium Persulphate [†]	200 μ l	200 μ l	200 μ l
TEMED [¶]	20 μ l	20 μ l	20 μ l

Table 2.8.2.1 Components of resolving gel (8%, 12% and 15%) used for Western blot analysis. [†] 0.1% (w/v)

STACKING GEL	QUANTITY FOR 4% GEL
REAGENT	
Protogel	1.3 ml
Protogel Stacking Buffer	2.5 ml
dH ₂ O	6.1 ml
Ammonium Persulphate [†]	50 µl
TEMED [¶]	10 µl

Table 2.8.2.2 Components of stacking gel (4%) used for Western blot analysis.

[†] 0.1% (w/v)

The gel casting unit was constructed according to the manufacturers' instructions. Glass plates were slotted into the clamp assembly and water-tightness tested to ensure no leaks. The resolving gel was prepared as detailed above, vortexed and poured to ~15 mm of the total height of the glass plates. To enhance polymerisation and reduce meniscus formation, ~3 mm of 70% isopropanol was carefully layered over the resolving gel. The gel was then left to set for ~30 min at RT. Once set, the surface of the resolving gel was gently washed with dH₂O and the stacking gel prepared. This gel solution was poured onto the surface of the resolving gel and wells created by inserting a comb (1.5 mm thickness, 10 teeth of 5 mm wide each) at an angle to minimise introducing air bubbles into the gel. Any loss of gel was immediately replaced. Once the stacking gel was set, the comb was removed vertically and wells were flushed vigorously with a syringe containing electrode buffer. Gels encased within the glass plates were transferred to a tank containing 1L of 1X electrode buffer and secured into place creating two separate reservoirs, allowing ionic movement only through the gels. Wells were flooded and any leakages between the two reservoirs were stopped. Samples were loaded using specific loading tips (Fisher, UK) and air bubbles expelled. Samples were resolved by electrophoresis.

2.8.3 Electrophoretic Transfer (Blotting)

Immobilon-PTM PVDF membranes (0.45 μm) were briefly immersed in 100% methanol, washed in dH₂O for 1 min then soaked in transfer buffer until use. For smaller sized proteins, Westran PVDF membranes (0.2 μm) were briefly immersed in 100% methanol and soaked in Transfer Buffer for 5 min. After ~1 hr, gels containing the resolved proteins were removed from the glass plate housing. Stacking gels were discarded and the resolving gels were soaked in transfer buffer for 10 min. Fibre pads and filter paper sets (2 sets per gel) were soaked in transfer buffer for 10 min. Two pieces of filter paper were laid over a saturated fibre pad and gel layered on to the filter paper (orientated to be identical to loading sequence) expelling all air bubbles. One piece of permeabilised Immobilon-PTM membrane was then layered over the gel, ensuring that no bubbles interrupted the contact between the gel and membrane. This was sandwiched between further layers of saturated filter paper and fibre pads. These stacks were transferred to transfer casings and placed into a tank containing 1L of transfer buffer. Proteins were transferred electrophoretically at a constant voltage of 100V for 1hr.

2.8.4 Primary Antibody Application

Following the transfer of proteins, membranes were removed from between the filter paper and fibre pads layers and trimmed to mark orientation and membrane number. Membranes were incubated with 10% non-fat milk solution diluted in 0.5% PBS (Tween 20 (0.1% (v/v),) and placed on an orbital shaker for either 1 hr at RT or overnight for ~14 hr at 4°C (conditions vary depending on the protein of interest). This procedure prevents the primary antibody binding directly to the PVDF membrane and further blocks non-specific binding of proteins to the membrane.

Following blocking, membranes were rinsed 3 times in PBS and once in 0.1% PBS-T. Primary antibody was then prepared in sufficient quantity to cover the surface of the membrane at a concentration of 0.1% PBS and 0.1% PBS-T and incubated in a 50 ml centrifuge tube for 1 hr at RT or 4°C overnight on an orbital shaker. After ~14 hr at 4°C, membranes were washed 3 times with PBS and once in PBS-T. Membranes were returned to the shaker for three 10 min washes in excess 0.1 % PBS-T.

2.8.5 Secondary Antibody Application

Secondary antibody was made up in 0.1% PBS/PBS-T. Membranes were incubated at RT for 1 hr on an orbital shaker (80-100 cycles/min). Following incubation with secondary antibody, membranes were then rinsed three times in 0.1% PBS/PBS-T with three further 10 min washes in excess 0.1% PBS-T.

2.8.6 Immunodetection of Antibody Labelled Proteins

The secondary antibody allows the detection of specific proteins by serving as a catalyst for luminol, a substrate that luminesces, which part of an ECL plus Western blotting detection system. The reaction is based on the oxidation of the cyclic diacylhydrazide luminol. Secondary antibody conjugated with horseradish-peroxidase (HRP) binds to the specific primary antibody that is attached to the protein of interest. Combined HRP and peroxide catalyses the oxidation of the lumigen PS-3 acridan substrate generating thousands of acridinium ester intermediates per minute (Figure 2.8.6.1). These intermediates react with peroxide under slightly alkaline conditions to produce a sustained, high intensity chemiluminescence.

The light exuding from the reaction can be visualised by application of photographic X-ray film or real-time digital photography. Following secondary antibody incubation and subsequent washes in PBS (120 mM, pH 7.6), membranes were ready for the immunodetection of proteins. Membranes were firstly placed protein side-up on saran wrap. ECL plus Solution A (Tris Buffer) was mixed with Solution B (Acridin Solution in Dioxane and Ethanol) at a 1/40 ratio to a volume required to cover each membrane (4 ml/membrane). This mixture was incubated on the membrane for 5 min at RT. Following incubation, ECL plus was discarded and the corner of the membrane blotted on tissue paper to remove excess ECL mixture. Membranes were then sandwiched between two sheets of clear plastic and excess ECL was removed with tissue paper and then placed into a film cassette. The filters were then exposed to photographic X-ray film (Kodak, UK) for varying times depending on the antibody used (1-60 min).

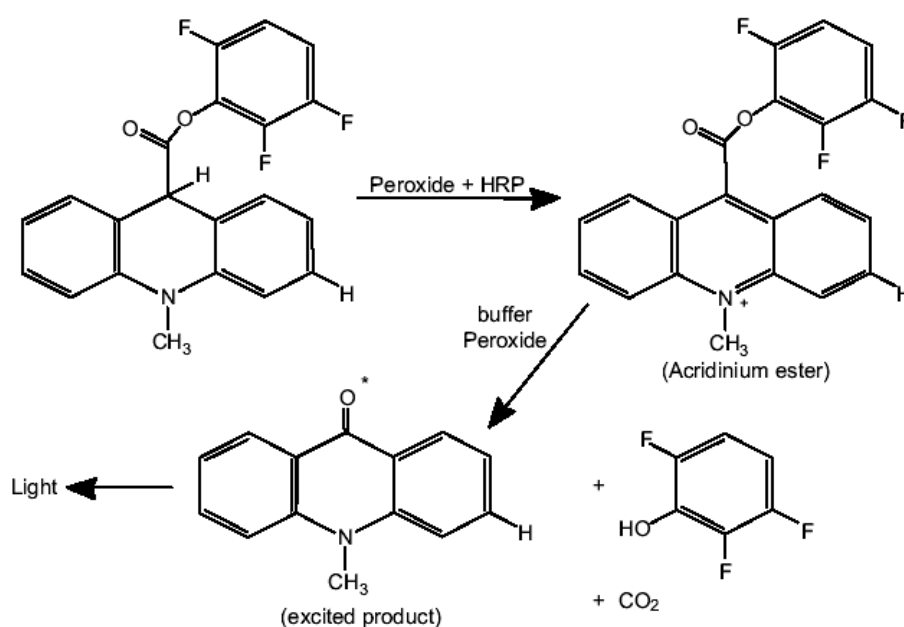


Figure 2.8.6.1 Chemiluminescent reaction of Lumigen PS-3 with horseradish-peroxidase (HRP) (Modified from Isacsson and Watermark 1974).

2.8.7 Quantification of Western Blot Protein Bands

Autoradiographs were quantified by gel blot analysis using UVP Gel Blot Analysis System and appropriate statistical analysis as detailed in individual results chapters

2.9 Extraction & Quantification of RNA.

2.9.1 RNA Isolation & Purification from Adipose Tissue for Quantitative PCR

RNA samples from adipose tissue and differentiated primary adipocytes were purified, quantified and processed according to the following procedures.

A column-based method (RNeasy Lipid Tissue Mini Kit for total mammalian mRNA isolation) was implemented to extract mRNA from the adipose tissue in accordance with manufacturer's instructions. This process included a deoxyribonuclease (DNase) digestion step with 5 µl DNase enzyme diluted in 5 µl reaction buffer to remove any contaminating genomic DNA. The tubes containing these reactants were incubated at standard RT for 15 min before the reaction was quenched through the addition of 5 µl of stop solution and immediate incubation at 70°C for 10 min. RNA was quantified with a spectrophotometer using a ratio of readings at a wavelength of 260 and 280 nm. Once quantified the isolated RNA samples were stored at -70°C.

2.9.2 Reverse Transcription of Isolated mRNA from Adipose Tissue

An aliquot containing 1 µg of mRNA from each sample was pipetted into a sterile microcentrifuge tube along with 0.5 µl of random hexamers mixed in equal quantities. To standardise, nuclease free water was then added to each aliquot to make up a final volume of 10 µl. Each reactant mixture was then incubated at 70°C for 10 min. Reverse transcriptase (RTn) master mix was then prepared (Table 2.9.2.1) in sufficient quantities to allow for the required number of RTn reactions. After a

thorough vortex, 10 μ l of master mix were added to each incubated sample of mRNA. Each sample was then vortexed, centrifuged briefly and heated at 37°C. After 1 hr, the reaction was quenched by a consecutive incubation at 95°C for 5 min to denature the enzymes. The resultant cDNA product was stored at -20°C for further use.

Contents of Reverse Transcription Master Mix	Concentration in Final 20 μ L Volume	Quantity Added (μ L)
Reverse Transcription 10X Reaction Buffer (Mg ²⁺⁺ free)	1x (v/v)	2.0
Mg ²⁺⁺ (25 mM)	5 mM	4.0
dNTPS (10 mM)	1 mM	2.0
AMV (20 U/ μ l)	0.5 u/ μ l (v/v)	0.5
RNasin (40 U/ μ l)	1 u/ μ l (v/v)	0.5
Nuclease free H ₂ O	5%	1.0

Table 2.9.2.1 The contents of the reverse transcription master mix. The final volume of cDNA sample is 20 μ l where 1 μ l equals 50 ng of single stranded cDNA.

2.9.3 Quantitative Real-Time PCR

The real-time PCR reaction was performed in 25 μ l volumes on 96 well plates, in a reaction buffer (Taqman universal PCR master mix), containing 3 mM Mn(Oac)₂, 200 pM dNTPs, 1.25 units ampliTaq gold polymerase, 1.25 units ampErase UNG, 100-200 nmol Taqman probe, 900 nmol primers and 25-125 ng cDNA. All reactions were multiplexed with the housekeeping gene for the 18S ribosomal subunit, provided as a pre-optimised control probe enabling data to be expressed in relation to an internal reference to allow for differences in RT-PCR efficiency. Data were obtained as the cycle number at which logarithmic PCR plots cross a calculated threshold line or the Ct value. In accordance of the manufacturer's guidelines Δ Ct values were determined (Δ Ct=Ct of the target gene minus Ct of the housekeeping gene).

Measurements were carried out in triplicate and the target gene probes were labelled with the fluorescent label FAM, and the housekeeping gene with the fluorescent label VIC. Reactions are as described in **Table 2.9.3.1**.

Thermal Cycler	Times and Temperatures			
	First Steps		1 of 44 cycles	
			Melt	Anneal/ Extend
ABI Prism 7700 Sequence Detector	Hold	Hold	Cycle	
	2 min 50°C	10 min 95°C	15 s 95°C	1 min 62°C

Table 2.9.3.1 RT-PCR reaction conditions as pre-set on the ABI 7700.

2.9.4 Data Handling & Statistical Analysis

For RNA analysis to exclude potential bias due to averaging data, which had been transformed through the equation $2^{-\Delta\Delta C_t}$, all statistics were performed at the ΔC_t stage. For both mRNA and protein findings, statistical analysis was undertaken using an unpaired Student's t-test. The threshold for significance was $p < 0.05$. Data in the text and figures are presented as mean \pm SD as noted in the relevant results chapters.

2.10 Enzyme Linked Immunosorbent Assay (ELISA)

The sandwich enzyme-linked immunosorbent assays (ELISAs) used was based on the reaction between an antigen and an antibody. In essence ELISAs involve the attachment of a capture antibody to a solid phase support. Samples containing known or unknown antigens are then added in a matrix or buffer that will minimize attachment to the solid phase. An enzyme-labelled antibody is then added for detection as illustrated in Figure 2.10.1.

All ELISA based assays applied in this thesis were performed using ready prepared commercially available kits. The sources of these kits are outlined in the relevant chapters. In all cases, both ELISA analyses were carried out in accordance of the manufacturers' instructions of the individual kits. All the corresponding CV, intra- and interassay values are given for each individual assay in the relevant chapters. Appropriate statistical analysis for ELISA was carried out using a statistical software package (SPSS for Windows, Version 14.0, Woking, UK).

ELISA Diagram

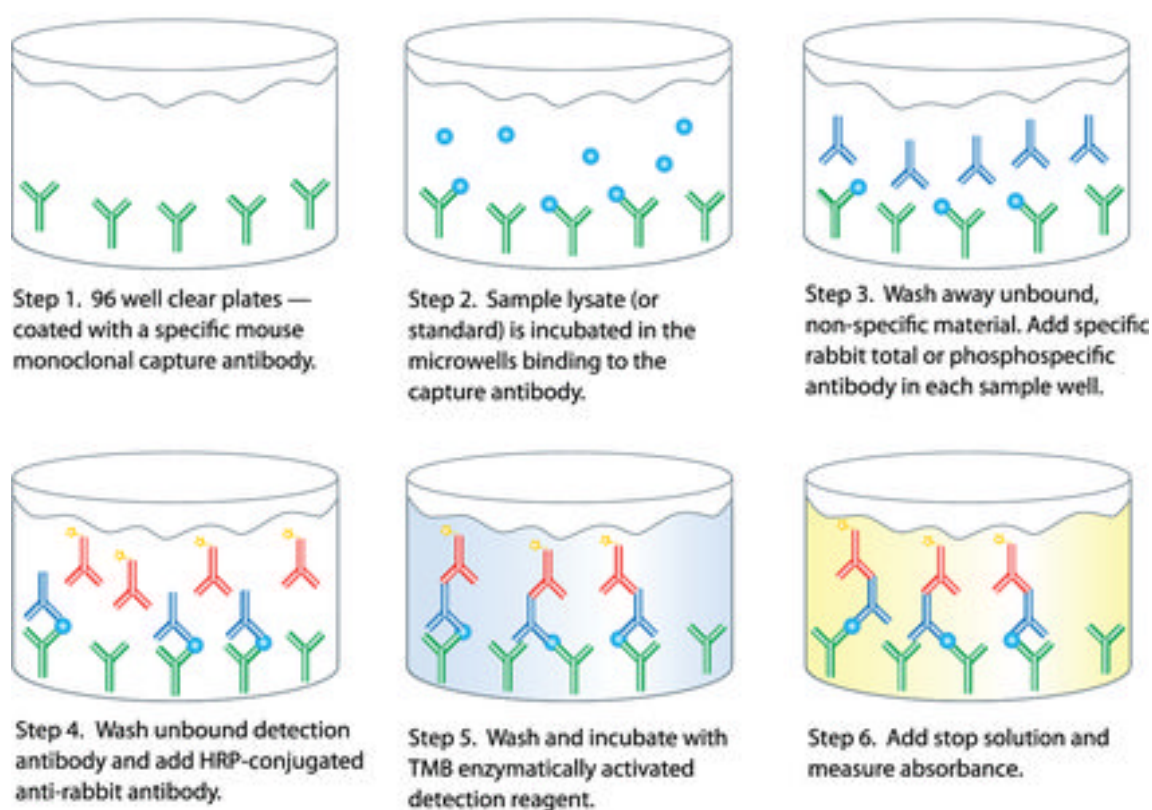


Figure 2.10.1 Schematic diagram depicting steps of ELISA protocol

Adopted from <http://www.millipore.com/catalogue/module/c77254>

Chapter 3

Characterisation of Differentiating Primary Human Abdominal Subcutaneous Preadipocytes

3.1 Introduction

Within AT, in addition to mature adipocytes, there are a variety of other cell types required for normal tissue function, including pre-adipocytes, leukocytes, macrophages and endothelial vascular cells. The main cell type within AT are adipocytes, unilocular cells which can range in diameter from 20-200 microns (μm) and have the capacity to increase their diameter of up to 20-fold, allowing them to increase in volume of up to several 1000-fold, in order to maximise energy storage (Fruhbeck *et al.*, 2001). Pre-adipocytes are undifferentiated precursor cells for adipocytes, and act as an adipocyte store in cases where hyperplastic growth is required, causing pre-adipocyte cells to fully differentiate into mature adipocytes. Endothelial cells form the tissue vasculature supplying blood to AT, and transporting proteins to and from the periphery. Leukocytes, particularly macrophages, reside within AT and are recruited to a site of inflammation to eliminate infected cells. The predominant immune cell within AT is the macrophage, constituting 5-10% of cells in lean subjects, and expanding to up to 40% of the AT cell population in individuals with a diet induced increase in adiposity (Weisberg *et al.*, 2003).

The maintenance of energy homeostasis requires a balance to be maintained between energy consumption and energy expenditure. When this balance can no longer be maintained e.g. via excessive energy intake in relation to energy expenditure, a 'positive energy balance' is created; this can result from insufficient physical exercise and excessive calorie intake. This excess energy is stored within white AT (Frayn *et al.* 1995; Gregoire and Smas, 1998), and can negatively impact upon the AT architecture. Adipocytes can rapidly grow in size (hypertrophy due to lipid uptake) and number (hyperplasia due to cell differentiation and proliferation). Both processes are regulated genetically and hormonally, and key genes are

involved in TG formation (lipogenesis), and TG breakdown (lipolysis) (Gregoire *et al.*, 1998).

Hyperplasia and hypertrophy are the processes by which AT is acquired shortly after birth (Gregoire *et al.*, 1998). The increase in AT mass through hyperplasia and hypertrophy is termed ‘adipogenesis’, a process which continues throughout adulthood. Hyperplastic adipocyte growth, or ‘proliferation’ occurs through ‘clonal expansion’ of preadipocytes and leads to formation of new precursor cell populations (Prins *et al.*, 1997). Hyperplastic growth and its control involve preadipocyte proliferation and differentiation from pluripotent stem cells (Rodriguez *et al.*, 2005). Such cells are capable of undergoing cellular ‘differentiation’ or ‘maturation’ and developing into a range of different cell types, including adipocytes, chondrocytes, osteocytes or myocytes (MacDougald and Mandrup; MacDougald and Mandrup, 2002).

The propensity of preadipocytes to proliferate and differentiate into different cell types is under tight regulation from adipogenic stimulatory factors, such as ‘insulin-like growth factor-1 receptors’ which act through insulin receptor substrate (IRS) molecules (Miki *et al.*, 2001), peroxisome proliferator activated receptor (PPAR)- γ (MacDougald and Mandrup) and free fatty acids (FFAs); adipogenic inhibitory factors, that inhibit hyperplastic growth, such as inflammatory cytokines, that include tumour necrosis factor- α (TNF- α), interleukin-6 (IL-6) (Skurker *et al.*, 2005; Maury and Brichard, 2010) interferon- γ , growth factors (MacDougald and Mandrup, 2002), and certain androgens (Dieudonne *et al.*, 2000).

The aim of this current chapter was to document that the abdominal subcutaneous preadipocytes, gained through elective surgery, undergo appropriate differentiation to be used for subsequent treatment regimens 15 days post-differentiation. These current studies examined lipid turnover, through glycerol release, and lipid accumulation, through oil red O

staining, to observe differentiation; photographic evidence was also taken to support the oil-red O data captured. Gene expression levels were assessed in the differentiating adipose cells and included adiponectin, PPAR γ , CCAAT enhancer-binding protein alpha (CEBP α) and perilipin. Final analysis examined protein secretion of adiponectin and leptin into the media from differentiating adipocytes, in order to substantiate that the abdominal subcutaneous pre-adipocytes were fully differentiated post 12 days.

The ultimate aim of this chapter was to characterise the differentiation of the human preadipocyte cells to show that the cells could be differentiated and their properties were consistent across all the differentiated cells. Fifteen days post-differentiation, these cells would be cultured in different treatment regimens for subsequent studies.

3.2 Materials & Methods

3.2.1 Adipose Tissue Isolation

AbSc AT from seven subjects was digested with collagenase (Worthington Biochemical, Reading, USA) as previously described in the methods chapter. In brief the isolated stromal fractions of human AbSc AT were routinely cultured into tissue culture flasks until confluent and then trypsinised to obtain cells to carry out the study. The pre-adipocytes from the same passage were grown in 6-well plates to confluence (100% cell plate cover) in DMEM/Ham's F-12 phenol-free medium (Invitrogen, Paisley, UK) containing 10% foetal-calf serum (FCS), penicillin (100 U/ml), streptomycin (100 µg/ml), and transferrin (5 µg/ml). At confluence, preadipocytes were differentiated in preadipocyte differentiation media (Promocell, Germany) containing biotin (8 µg/ml), insulin (500 ng/ml), dexamethasone (400 ng/ml), IBMX (44 µg/ml), L-Thyroxine (9ng/ml) and a thiazolidinedione, Ciglitazone (3µg/ml) for 72 hrs. After this period, the differentiating cells were grown in nutrition media containing DMEM/Ham's F-12, 3% FCS, d-biotin (8 µg/ml), insulin (500 ng/ml) and Dexamethasone (400 ng/ml) until fully differentiated (15 days) up until day 21.

3.2.2 Lipolysis Studies in Differentiated Pre-adipocytes

In brief, conditioned media collected during the course of differentiation of the preadipocytes was assessed for glycerol production as a measure of lipolysis (µM/mL) using a commercially available colorimetric kit (Randox Laboratories, Co. Antrim, UK). Cell culture medium samples were analysed for free glycerol content using the commercial glycerol free assay reagent and glycerol standards according to manufacturer's protocol. Briefly, to 25ul of

conditioned media in each well, 200ul of free glycerol reagent was added per well, incubated for 15mins at RT and absorbance read at 540nm. Glycerol release in the samples was calculated from the reference curve generated from known concentrations of glycerol.

3.2.2.1 Lipid Staining of Differentiated Preadipocytes

Oil red O is a fat soluble dye, and can be used as an effective reagent in the assessment of the extent of preadipocyte differentiation, as it targets and stains TG and lipids within the cell. Subsequent release of oil red O, following staining, can enable assessment of lipid accumulation within the culture. Lipid staining was performed using a modified method by Mcvean *et al*, (McVean *et al.*, 1965). Briefly, during defined intervals, differentiated preadipocytes were washed with Hanks' Balanced Salt Solution (HBSS), fixed for 1hr in 10% formalin, washed with water and stained with 0.3% Oil Red O (Gurr Ltd., London, UK) for 15 min at room temperature. After thorough washings with water, microscopic images were taken to visualize pink to red oil droplets staining differentiated cells. Then, after evaporation of excess water, oil red was extracted in isopropyl alcohol (Fisher Scientific Ltd., Loughborough, UK) and the absorbance was monitored at 520 nm using microplate reader for semi-quantitative determination of lipid accumulation.

3.2.2.2 Leptin & Adiponectin Secretion in Differentiating Human Preadipocytes

Conditioned media samples were collected during the course of differentiation and assayed for leptin and adiponectin secretion, as markers of differentiation, using respective enzyme-linked immunosorbant assay (ELISA) kits (leptin, Millipore; adiponectin Millipore, Hertfordshire, UK). Conditioned media were analysed using a solid phase ELISA, the leptin

ELISA had a sensitivity limit of 0.5 ng/ml, CV intra-assay 2.6-4.6% and inter-assay variability was 1.4-4.9%; adiponectin's assay limit was 0.78ng/ml, CV intra-assay 7.4% and inter-assay variability was 2.4-8.4%. Methodology for a standard ELISA is given in the materials and methods.

3.2.2.3 Extraction of differentiating Preadipocytes for RNA Extraction and Quantitative RT-PCR

RNA was extracted from treated isolated adipocytes using the GenElute total mammalian RNA kit (Sigma-Aldrich Corp.), which included a deoxyribonuclease digestion step to remove any contaminating genomic DNA. RNA (1µg) from each sample was reverse transcribed using Superscript (Invitrogen, Paisley, UK) and random hexamers in 20µl reaction volumes, according to the manufacturer's instructions. The mRNA levels were determined using an ABI 7500 real-time PCR Sequence Detection system (PE Applied Biosystems, Warrington, UK), as previously described (McTernan *et al.*, 2002). The reactions were performed in 25µl volumes on 96-well plates in reaction buffer containing TaqMan Universal PCR Master Mix (PE Applied Biosystems) and commercially available specific gene expression assays (PE Applied Biosystems).

3.2.2.4 Quantitative Primer and Probe Sequences for Adiponectin, PPAR γ , Perilipin and CEBP α , Genes were analyzed using Commercially Available Gene Expression Assays

All reactions were multiplexed with the housekeeping gene 18S, provided as a preoptimized control probe (PE Applied Biosystems, Warrington, UK), enabling data to be expressed in relation to an internal reference to allow for differences in RT efficiency. Data were obtained

as Ct values according to the manufacturer's guidelines (the cycle number at which logarithmic PCR plots cross a calculated threshold line) and were used to determine ΔCt values ($\Delta\text{Ct} = \text{Ct of the target gene minus Ct of the housekeeping gene}$). Measurements were carried out on at least three occasions for each sample. To exclude potential bias due to averaging data that had been transformed through the equation $2^{-\Delta\text{Ct}}$, all statistics were performed at the ΔCt stage.

3.2.3.1 Statistical analysis

For analysis of conditioned media and gene expression data, statistical analysis was undertaken using unpaired t tests unless otherwise stated, where data were analysed using nonparametric tests. The threshold for significance was $P < 0.05$. The data in the text and figures are presented as the mean \pm SEM unless otherwise stated.

3.3 Results

3.3.1. Influence of Differentiation on Lipolysis

To assess the effect of differentiation over time in preadipocytes, studies examined glycerol release as a marker of lipolysis in cultured AbSc adipocytes. Glycerol release was examined at day 0, 4, 9, 14, 18 and 21 post differentiation. Glycerol release increased over time as lipid content within the differentiating cells increased from baseline allowing a significant turnover of glycerol (Day 0: $4.23 \pm (\text{SD}) 1.3 \mu\text{g/ml}$; Day 4: $4.06 \pm 0.1 \mu\text{g/ml}$; Day 9: $9.89 \pm 4.2 \mu\text{g/ml}^*$; Day 14: $10.99 \pm 2.4 \mu\text{g/ml}$; Day 18: $14.63 \pm 3.5 \mu\text{g/ml}^{**}$; Day 21: $15.28 \pm 4.5 \mu\text{g/ml}^{**}$; p-values: $*p < 0.05$, $**p < 0.01$, $n=4$; **Figure 3.3.1.1**).

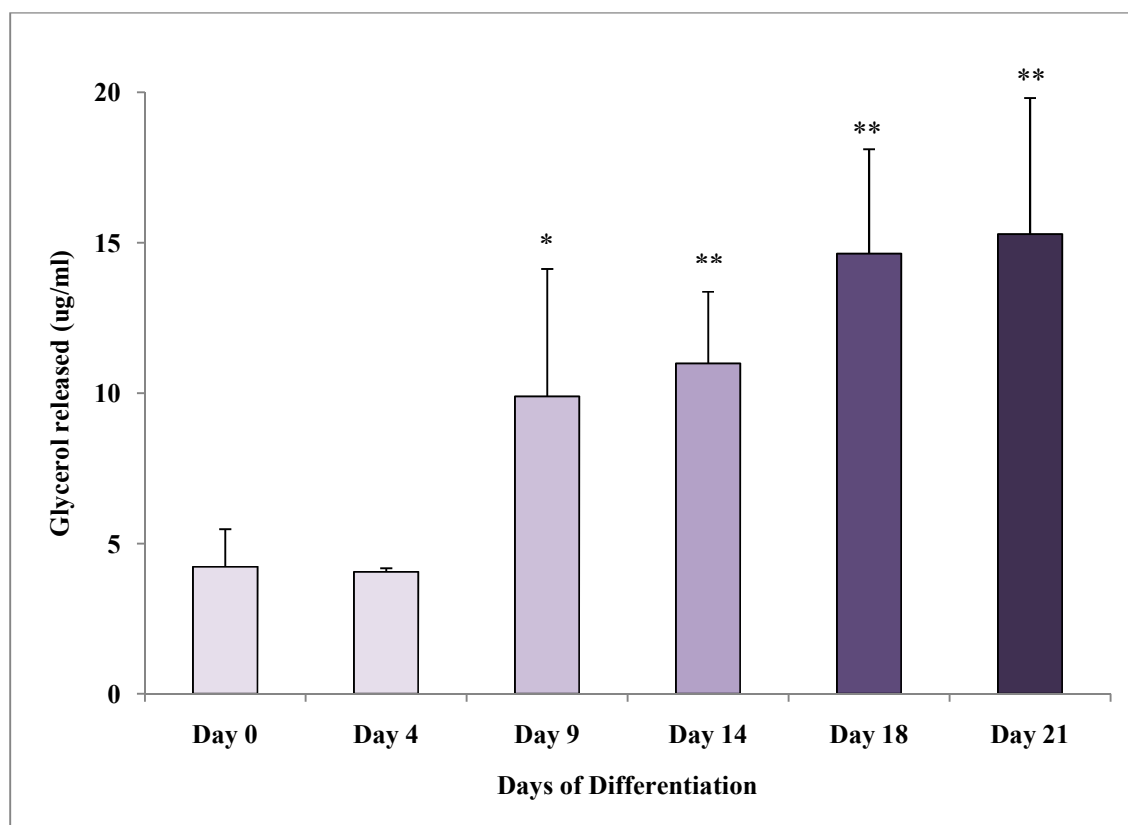


Figure 3.3.1.1 Measurement of glycerol release from differentiation of AbSc preadipocytes.

This figure highlights the increase in glycerol release over time, with significant changes noted when comparing day 0 with days 9, 14, 18 and 21 (p-values: * $p < 0.05$, ** $p < 0.01$).

3.3.2 Lipid Staining of Differentiated Preadipocytes

Oil red O release from lipid stained differentiated adipocytes was measured on the same days as the glycerol release studies and changes noted using a spectrophotometer, with absorbance defined as optical density units ODU at wavelength 520nm. There was a significant increase in lipid accumulation over time compared with baseline (Day 0: $0.075 \pm (SD) 0.01$ ODU; Day 4: 0.080 ± 0.016 ODU; Day 9: 0.099 ± 0.02 ODU; Day 14: 0.182 ± 0.06 ODU**; Day 18: 0.159 ± 0.01 ODU**; Day 21: 0.169 ± 0.002 ODU**; p-values: ** $p < 0.01$, $n=4$; **Figure 3.3.2.1**).

In addition to the spectrophotometric analysis of differentiation by oil-red O photographs were taken on selected days of differentiation as a visual sign of morphological changes in the preadipocytes as they accumulated lipids (**Figure 3.3.2A-D**).

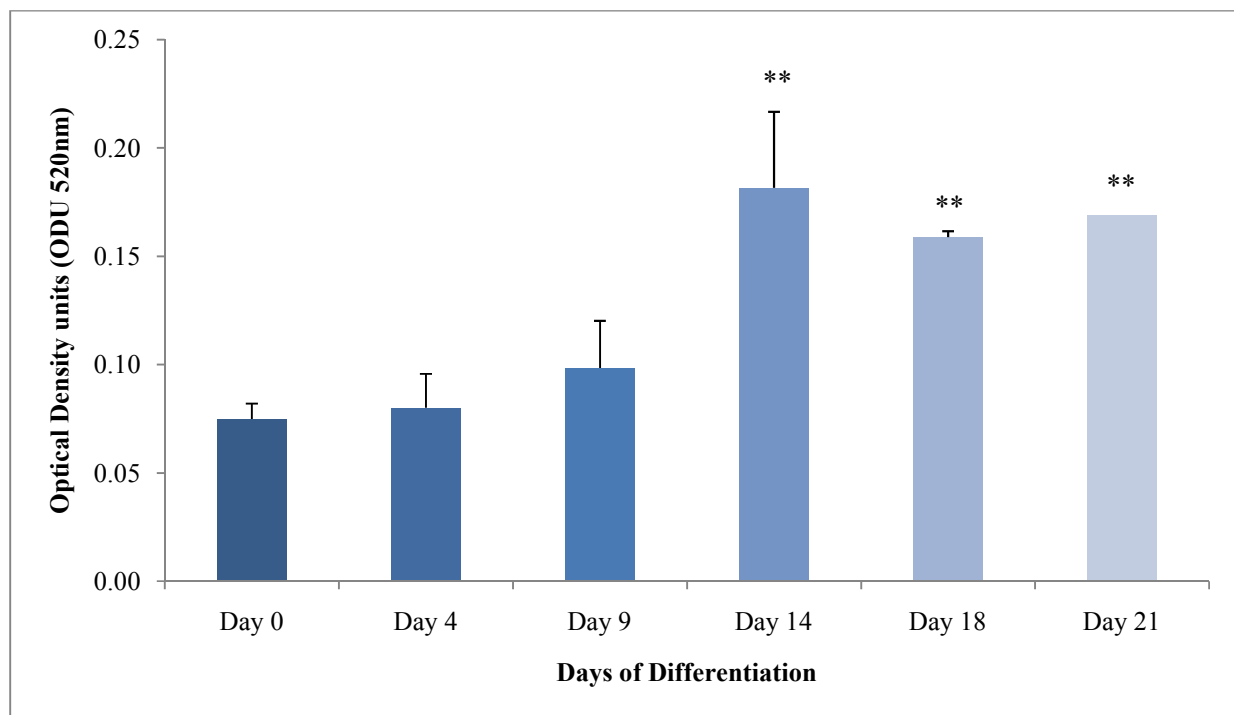


Figure 3.3.2.1 Measurement of oil-red O from Abd Sc differentiation of preadipocytes.

This figure highlights the increase in lipid accumulation as measured by oil-red O staining (520nm) of lipid over time, with significant changes noted when comparing day 0 with days 14, 18 and 21 (p-values: **p<0.01).

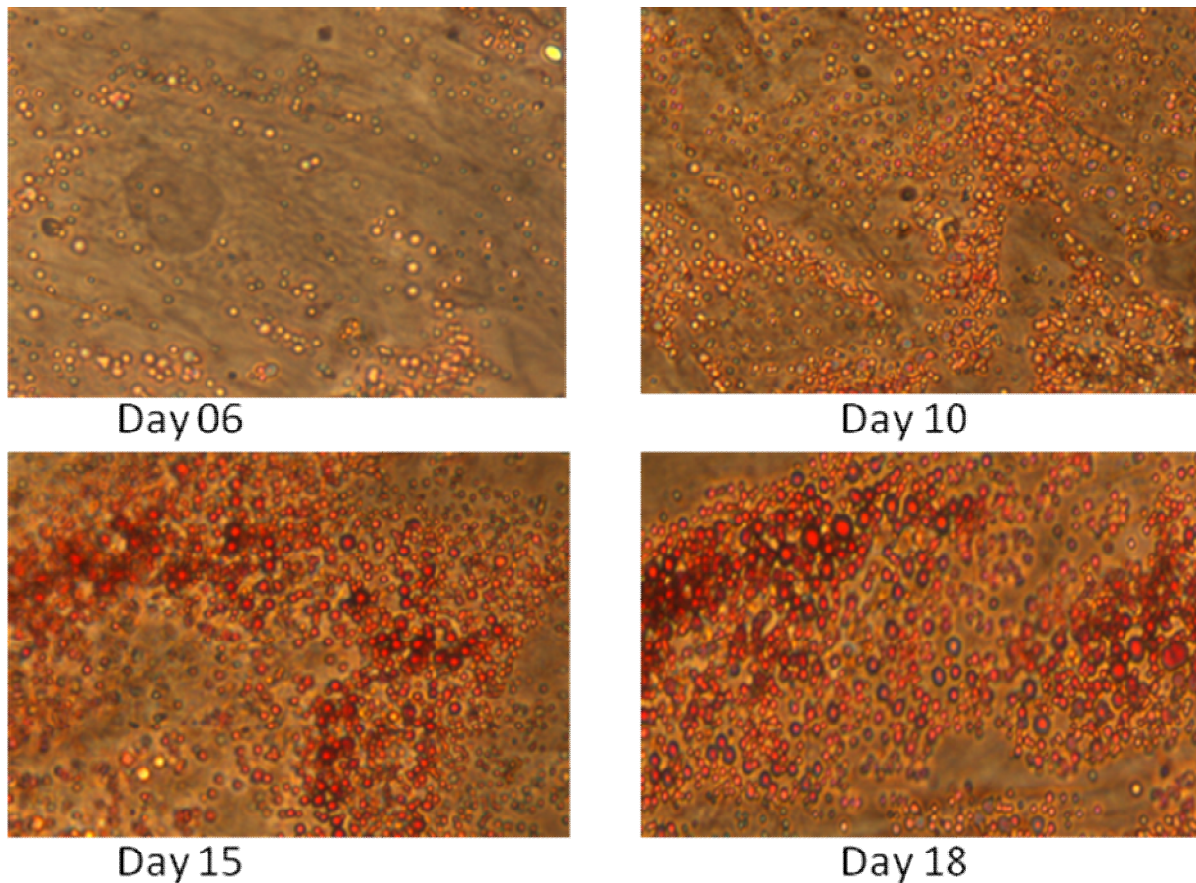


Figure 3.3.2.2 Photographic representation of lipid accumulation in differentiating AbSc preadipocytes. The photographs show the oil red O stained adipocytes from day 6-day 18 (magnification x40).

3.3.3 Leptin & Adiponectin Secretion in Differentiating Human Preadipocytes

Leptin was measured in conditioned media derived from differentiating AbdSc preadipocytes cultured in differentiation media, as defined in the methods and materials of this chapter. From analysis of the data comparing baseline Day 0 leptin secretion, leptin levels increased over the period of differentiation to a maximal level as denoted at Day 18 and Day 21 (Day 0: 0.35 ± 0.039 ng/ml; Day 4: 0.14 ± 0.045 ng/ml; Day 9: 4.51 ± 1.5 ng/ml^{**}; Day 14: 6.46 ± 0.25 ng/ml^{**}; Day 18: 10.73 ± 2.29 ng/ml^{**}; Day 21: 10.56 ± 0.94 ng/ml^{**}; p-values: ^{**} $p < 0.01$, p-values:, n=7; Figure 3.3.3.1).

Adiponectin was also measured in conditioned media as a marker of differentiation. From analysis of the data comparing baseline Day 0 adiponectin secretion, adiponectin levels increased over the period of differentiation to a maximal level as denoted at Day 9 with subsequent days reducing levels of adiponectin secretion, although still above day 0 secreted levels (Day 0: $0.199 \pm (\text{SD}) 0.03$ ng/ml; Day 4: 0.22 ± 0.065 ng/ml; Day 9: 0.47 ± 0.069 ng/ml**; Day 14: 0.36 ± 0.01 ng/ml**; Day 18: $0.335 \pm 0.097 \pm$ ng/ml*; Day 21: 0.36 ± 0.086 ng/ml*; p-values: * $p < 0.05$, ** $p < 0.01$, p-values:, n=7; Figure 3.3.3.2).

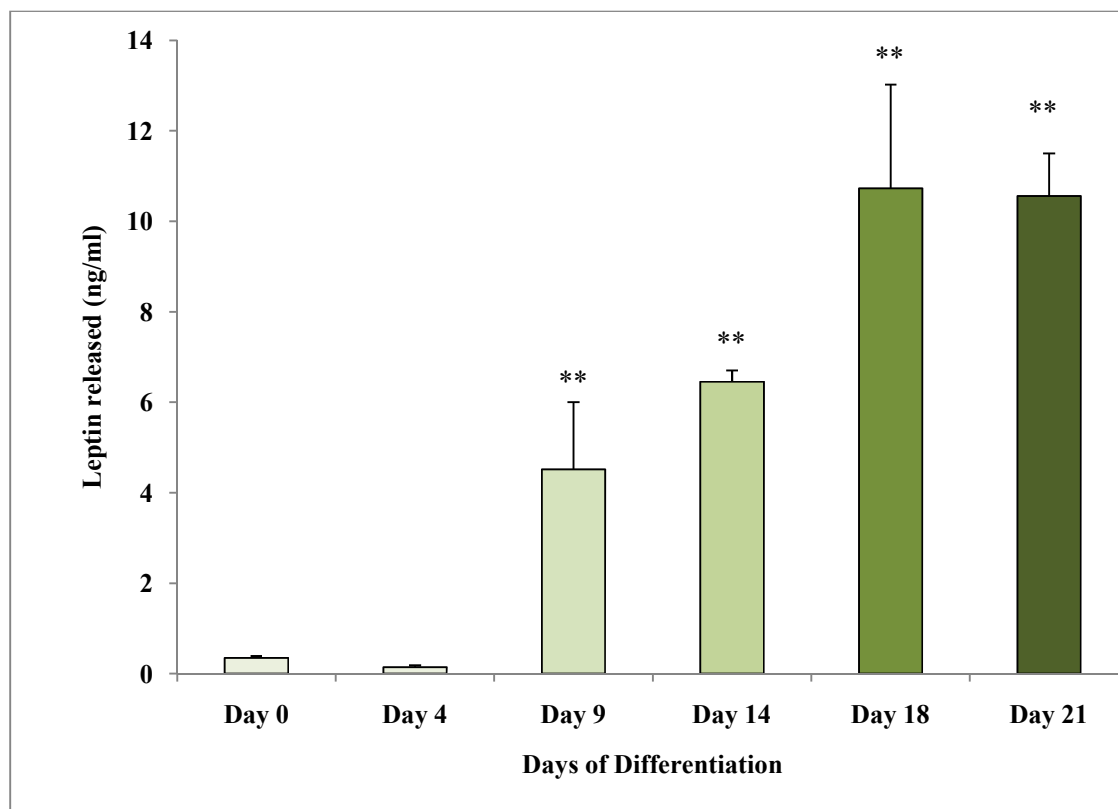


Figure 3.3.3.1 Secretion of leptin from differentiation of AbSc preadipocytes over time.

This figure highlights the increase in leptin secretion over time, with significant changes noted comparing day 0 with days 9, 14, 18 and 21 (p-value: ** $p < 0.01$).

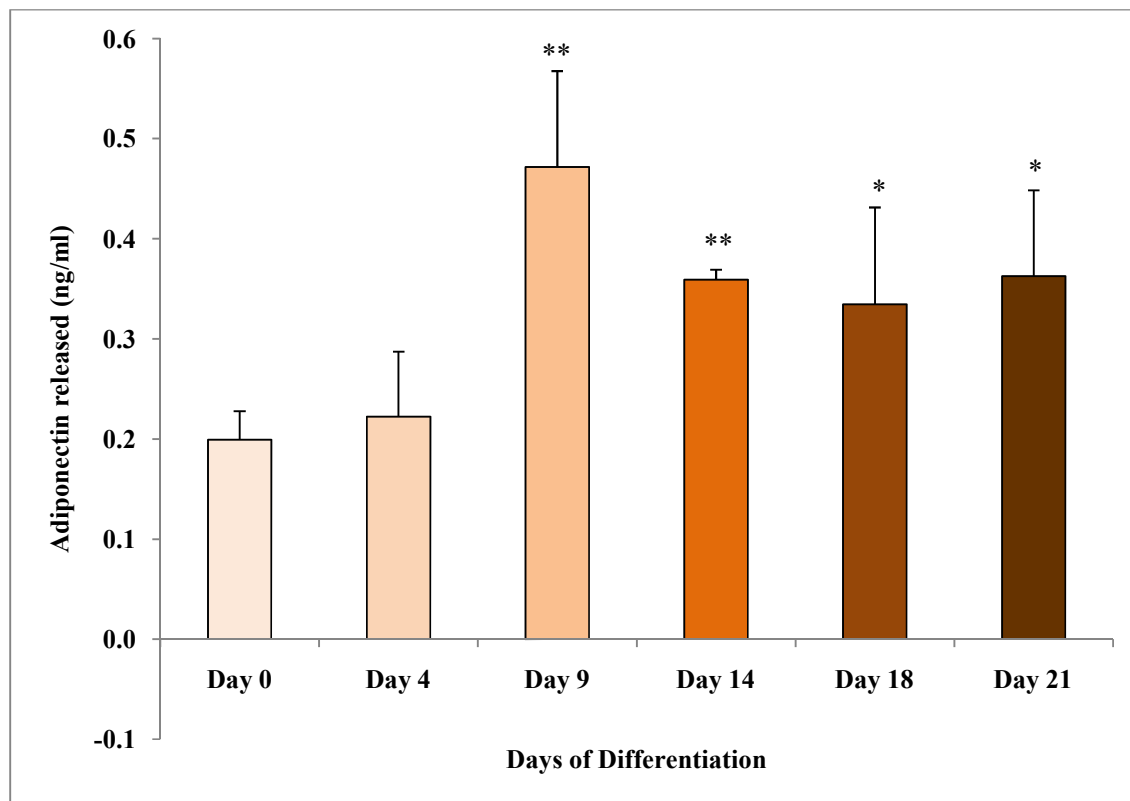


Figure 3.3.3.2. Secretion of adiponectin from AbSc differentiation of preadipocytes over time. This figure highlights the increase in adiponectin secretion over time, with significant changes noted comparing day 0 with days 9, 14, 18 and 21 (p-value: * $p < 0.05$, ** $p < 0.01$).

3.3.4 Gene Expression of Markers of Differentiation

Four gene expression markers of differentiation, adiponectin, PPAR γ , perilipin and CEBP α , were utilised to examine the changes in their mRNA levels during differentiation, in order to affirm the differentiation of the AbSc preadipocytes, in accordance with previous studies. **Table 3.3.4.1** shows the ΔC_t changes during differentiation ($n=7$); whilst Figure 3.3.4.2. A-D shows the relative fold expression with day zero given an arbitrary value of 1.

Days	$\Delta Ct \pm SEM$			
	Adiponectin	CEBP α	PPAR γ	Perilipin
0	25.56 \pm 0.05	28.22 \pm 0.76	19.78 \pm 1.41	28.16 \pm 0.46
3	22.06 \pm 0.54	23.64 \pm 1.49	17.38 \pm 0.46	18.99 \pm 0.81
6	22.75 \pm 1.66	23.93 \pm 2.24	16.64 \pm 1.49	17.75 \pm 1.64
10	23.9 \pm 1.01	25.53 \pm 1.24	18.11 \pm 0.66	20.63 \pm 0.92
12	24.12 \pm 0.54	24.71 \pm 0.21	18.04 \pm 0.04	18.68 \pm 0.89
15	23.23 \pm 1.77	24.4 \pm 0.95	17.79 \pm 0.74	19.89 \pm 1.05
18	23.33 \pm 0.86	24.48 \pm 0.68	17.69 \pm 0.35	20.05 \pm 0.76
25	24.34 \pm 0.21	25.52 \pm 0.04	18.78 \pm 0.17	23.25 \pm 0.29

Table 3.3.4.1 The gene expression profile for adiponectin, CEBP α , PPAR γ and Perilipin during differentiation. The gene expression data show the $\Delta Ct \pm (SEM)$ changes over time (measured as 24 hr period, Day 0-25).

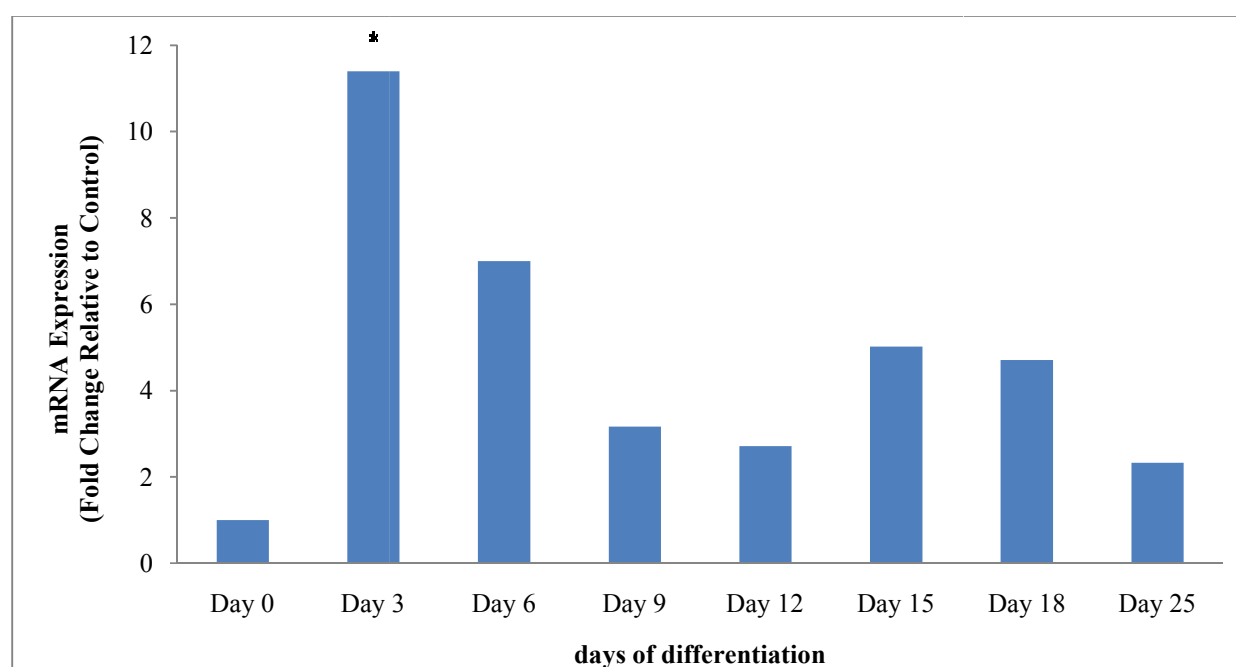


Figure 3.3.4.2 Adiponectin mRNA expression over-time in differentiating AbSc preadipocytes. This figure shows the relative fold change in adiponectin mRNA expression, with Day 0 taken as 1 and all others represented as a fold change relative to this over the days of differentiation. All data, including SEM, are highlighted in Table 3.3.4.1, which shows the $\Delta Ct \pm (SEM)$ changes over time (measured as 24 hr period, Day 0-25; P-value: * $p < 0.05$).

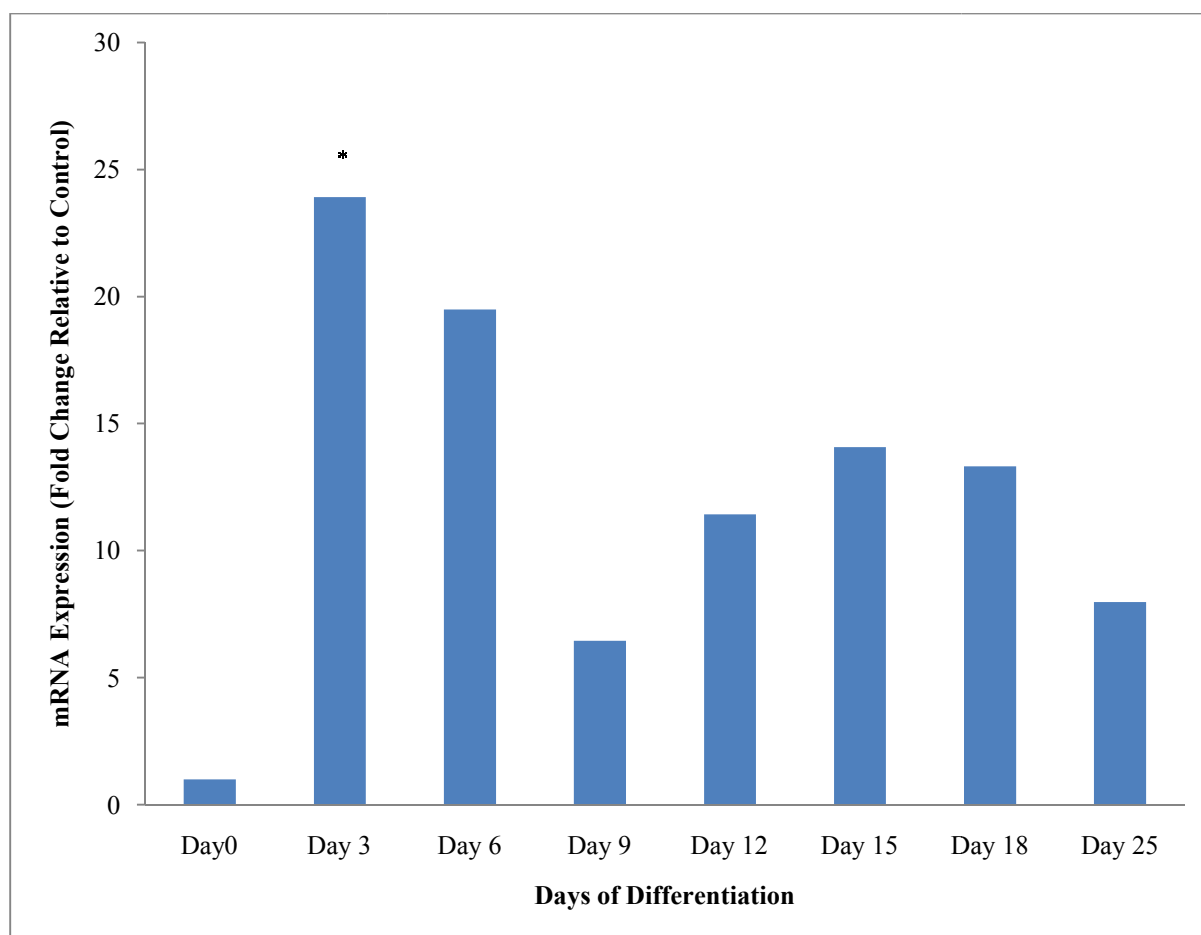


Figure 3.3.4.3. CEBP α mRNA expression over-time in differentiating AbSc preadipocytes.

This figure shows the relative fold change in CEBP α mRNA expression, with Day 0 taken as 1 and all others represented as a fold change relative to this over the days of differentiation. All data including SEM are highlighted in the above Table 3.3.4.1, which shows the $\Delta\text{Ct} \pm (\text{SEM})$ changes over time (measured as 24 hr period, Day 0-25; P-value: * $p < 0.05$).

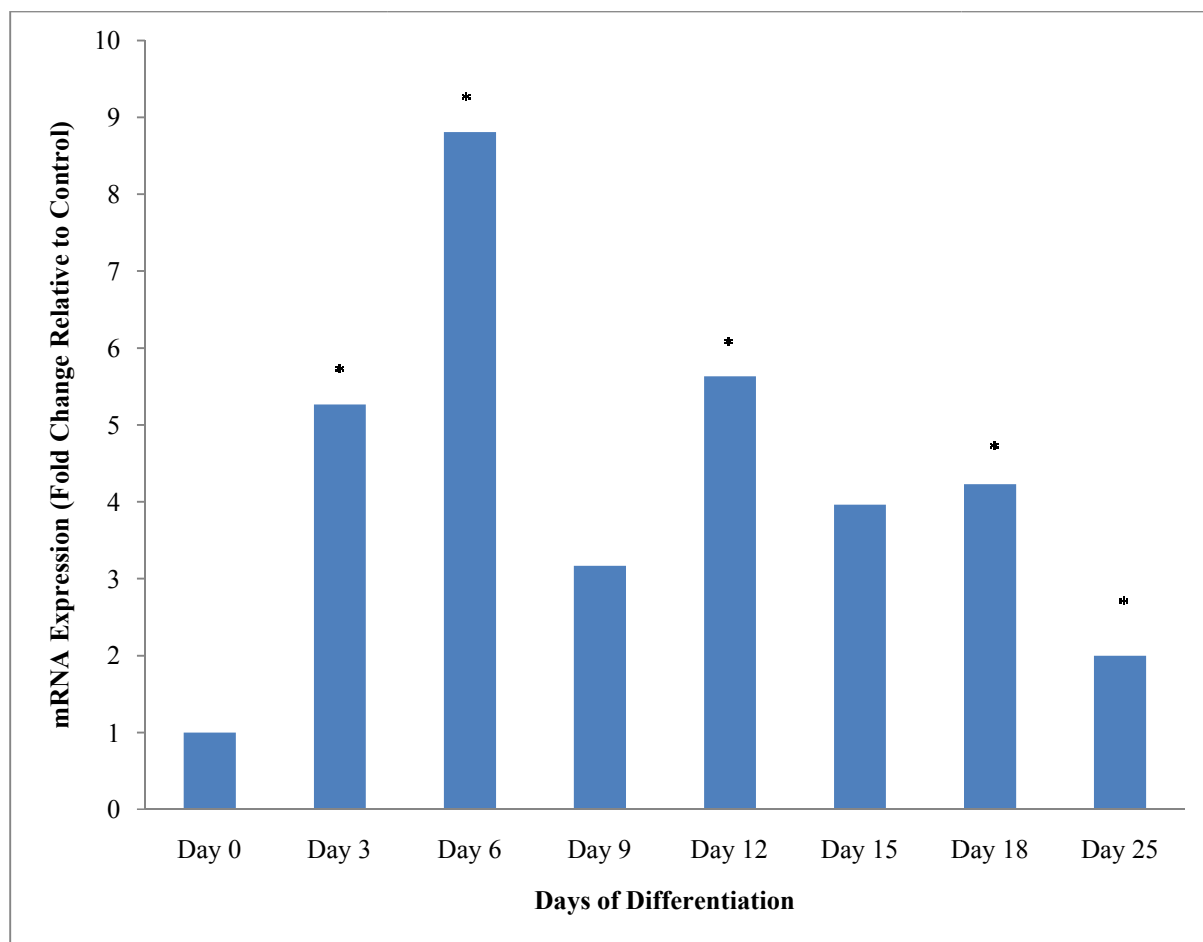


Figure 3.3.4.4. PPAR γ mRNA expression over-time in differentiating AbSc preadipocytes.

This figure shows the relative fold change in PPAR γ mRNA expression, with Day 0 taken as 1 and all others represented as a fold change relative to this over the days of differentiation. All data, including SEM, are highlighted in the above Table 3.3.4.1 which shows the $\Delta C_t \pm (SEM)$ changes over time (measured as 24 hr period, Day 0-25; P-value: * $p < 0.05$).

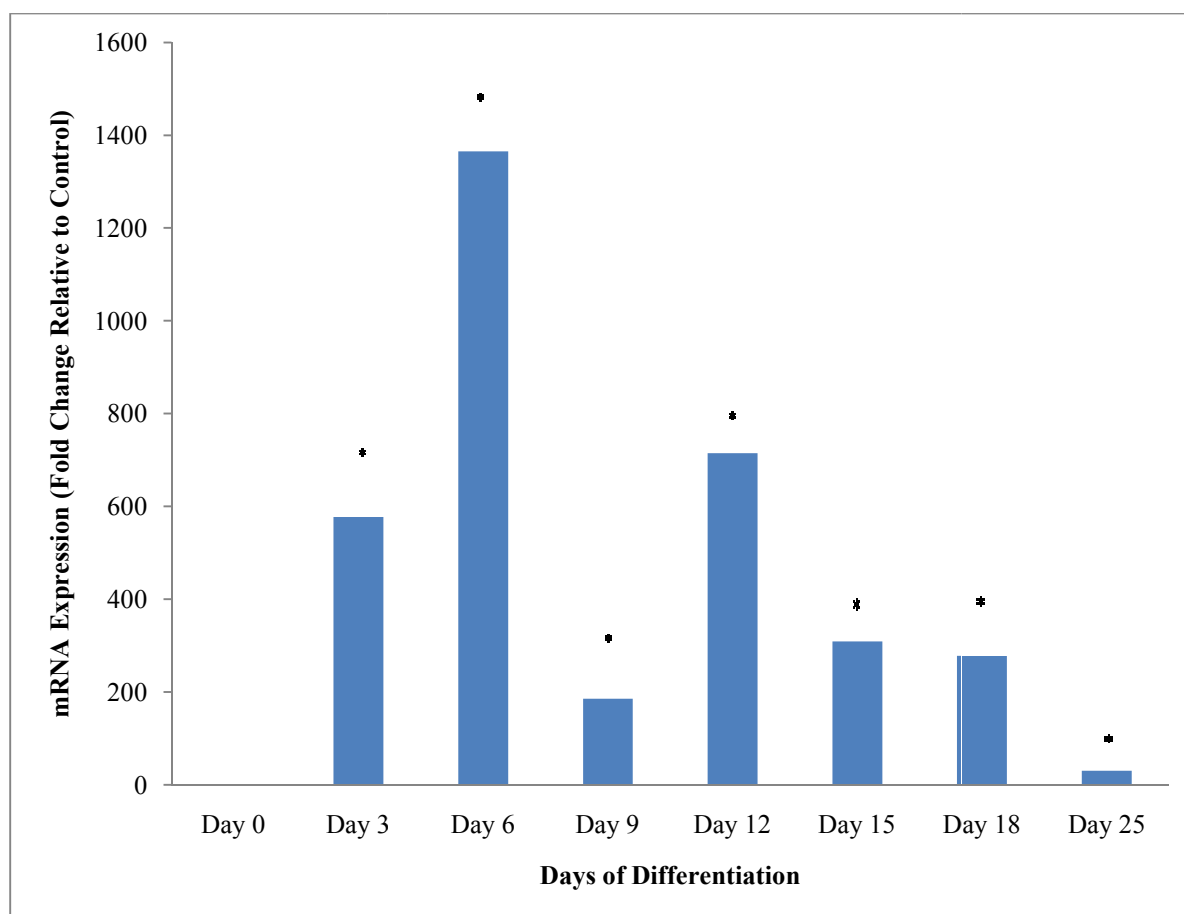


Figure 3.3.4.5. Perilipin mRNA expression over-time in differentiating AbSc preadipocytes.

This figure shows the relative fold change in Perilipin mRNA expression, with Day 0 taken as 1 and all others represented as a fold change relative to this over the days of differentiation. All data, including SEM, are highlighted in the above Table 3.3.4.1 which shows the $\Delta\text{Ct} \pm (\text{SEM})$ changes over time (measured as 24 hr period, Day 0-25; P-value: * $p < 0.05$).

3.4 Discussion

To date, there has been more than 60 papers examining the role of human preadipocyte differentiation and factors that can affect lipolysis, lipogenesis, as well as mRNA and protein expression and secretion of adipokines (Deng *et al.*, 2006; Dicker *et al.*, 2007; Kappes and Loffler, 2000; Marshak and Greenwalt, 2007; Payne *et al.*, 2007; Tian *et al.*, 2010). These papers have specifically concentrated on what mechanisms are affected by the perturbation of the differentiating system and how genes alter.

Early papers examining differentiation investigated whether lipolytic action was a function of differentiation or whether the rate of lipolysis can predict the state of preadipocyte differentiation. Such studies induced lipolysis using noradrenaline which was noted to increase significantly until day 12 of differentiation. However changes in mRNA expression of lipolysis related genes (such as hormone sensitive lipase (HSL), adipocyte triglyceride lipase, the α 2-and β 1-adrenoceptors, perilipin and fatty acid binding protein) all appeared to reach maximal effect much earlier during differentiation, specifically by day 8 (Dicker *et al.*, 2007). The studies presented here, examining perilipin and PPAR γ mRNA expression, identified a similar pattern with a significant increase in mRNA expression from baseline up until day 6 and then a subsequent and substantial drop that appeared to increase slightly after day 9 but never reached the marked increase in mRNA expression observed on day 6. Additionally, as the cells were given a TZD to drive differentiation, it was clearly noted that PPAR γ responded to this stimulus – thus substantiating previous findings that PPAR γ plays an important role in lipid metabolism, as well as insulin sensitivity, during differentiation of preadipocytes (Deng *et al.*, 2006). Previous studies have highlighted that expression of the HSL gene is up-regulated by PPAR γ and PPAR γ agonists (rosiglitazone and pioglitazone) in cultured hepatic cells and differentiating preadipocytes (Deng *et al.*, 2006).

Taken together, these mRNA data affirm previous findings on differentiation. In subtle contrast, functional analysis of glycerol release and oil-red O staining highlighted that lipid accumulation continued despite a reduction in lipid related genes, as demonstrated previously by Dicker and co-workers (Dicker *et al.*, 2007).

These current studies also examined mRNA expression of CAAT/enhancer-binding protein alpha (C/EBP alpha) which, along with C/EBP beta, has previously been shown to regulate Diacylglycerol acyltransferase 2 (DGAT2) expression during adipogenesis. DGAT2 is important as it catalyzes the final step of TG synthesis and therefore is important in adipogenesis promoting lipid accumulation. Previous analysis using both isolated murine preadipocytes and 3T3-L1 cells has shown that the temporal pattern of DGAT2 expression closely mimicked that of genes whose expression is regulated by the C/EBP family, with notable changes in C/EBP α and β expression during adipogenesis. This present study, again, highlighted a substantial reduction in CEBP α mRNA expression after day 6 that slowly increased over subsequent days (Dicker *et al.*, 2007; Payne *et al.*, 2007).

In addition to examining mRNA expression during differentiation, secretion of adipokines was also examined. These studies utilised conditioned media to assess the secretion of leptin and adiponectin by ELISA. Previous studies examining leptin secretion as adipogenesis progresses noted its substantial increase at the later stages of differentiation. Recent studies have also begun to capture the gene expression data as assessed by microarray and real-time PCR, to use bioinformatics algorithms to predict the secretome of differentiating preadipocytes, with the most significant adipokines confirmed at the protein level using western blots or ELISA assays. This study, as well as highlighting a novel candidate, matrix gla protein (MGP), which was upregulated (approximately 30-fold) during adipogenesis, also re-affirmed leptin release (approximately 50-fold increase (Mutch *et al.*,

2009). Such changes in leptin secretion in this present chapter align with Mutch's findings along with previous reports of leptin level changes during differentiation noted by others (McTernan, 2003). Furthermore the data in this chapter, examining adiponectin secretion, is in accordance with previous findings identifying a substantial up regulation of adiponectin mRNA and protein secretion post day 3 (Kappes and Loffler, 2000; Mutch *et al.*, 2009; Tian *et al.*, 2010).

To gather as much data across the individual preadipocyte sample several time points were investigated during differentiation and samples taken for either mRNA, oil red O, protein expression or glycerol release; where feasible samples were collected from the same time point in essence to maximise the data from each individual cultured preadipocyte cell sample (Deng *et al.*, 2006; Dicker *et al.*, 2007; Kappes and Loffler, 2000; Marshak and Greenwalt, 2007; Payne *et al.*, 2007; Tian *et al.*, 2010). This meant the same preadipocyte cell samples were grown to assess a variety of measurements over the course of the differentiation, rather than differentiating preadipocytes cells for one particular assay each time. It should be stressed though that this system was designed to work on a small scale to evaluate the effects on cellular mechanisms post differentiation and not to produce a high rapid throughput system, as has been established by others previously (Marshak and Greenwalt, 2007).

In summary, this current chapter has examined a variety of differentiation markers from lipolysis, adipogenesis, mRNA and protein data, as well as pictorial morphological changes during differentiation to highlight that, under the current culture system, the preadipocytes undergo differentiation comparable to previous studies. Following differentiation the cells were subsequently utilised for the studies as outlined in the following chapters, where post-differentiation cellular mechanisms of ER stress were investigated.

Chapter 4

The Effect of Hyperglycaemia on ER Stress and the Alleviating Effect of Salicylates on Primary Human Differentiated Abdominal Subcutaneous Adipocytes

4.1. Introduction

Obesity-associated inflammation is a key contributory factor in the pathogenesis of type 2 diabetes mellitus (T2DM) and cardiovascular disease (CVD) but the fundamental mechanisms responsible for activating innate immune inflammatory pathways and insulin resistance are currently unclear. Cardiovascular disease (CVD) is the main cause of morbidity and mortality in type 2 diabetes-related costs. In the diabetic population, the relative risk of coronary heart disease (CHD) is 1.5 to 1.7 in males and 1.7 to 4.0 in females (Charbonnel & Del Prato, 2003). The relationship between diabetes and cardiovascular events is so stringent that the American Diabetes Association has identified diabetes as a cardiovascular disease. Postprandial hyperglycaemia (PPG) is an important contributing factor in the development of CVD in people with T2DM (Ceriello, 2005). Two recent studies have confirmed that postprandial hyperglycaemia is an independent risk factor for CVD in type 2 diabetes (Hanefeld *et al*, 1996; Cavalot *et al*, 2006). Intervention studies have also supported the relevance of PPG in the development of CVD including silent myocardial infarction (Chiasson *et al*, 2002; Chiasson *et al*, 2003; Zeymer *et al*, 2004).

Therefore whilst clinically relevant, the mechanisms for such occurrences appear to reach further beyond cellular dysfunction of endothelial cells in response to PPG and may also impact on adipose tissue (AT). As such a new emerging area has begun to examine the link between the impact of obesity-induced inflammation, insulin resistance and increased stress of the endoplasmic reticulum (ER) (Hotamisligil, 2007; Gregor & Hotamisligil, 2007b).

As discussed fully in chapter 1 the ER represents a highly dynamic organelle with a central role in lipid and protein biosynthesis (Ozcan *et al*, 2004). It is extremely sensitive to alterations in homeostasis, and proteins formed in the ER may fail to attain correct structural conformation during pathological nutrient excess. Accumulation of misfolded proteins in the ER causes ER stress and activation of unfolded protein response (UPR) (Harding *et al*, 2002), which under normal circumstances alleviates ER stress, restores ER homeostasis and prevents cell death. One of the factors known to elicit cellular stresses include hyperglycaemia, however to date the effect of this on ER stress in differentiated primary human adipocytes is unknown (Cnop *et al*, 2005; Nakatani *et al*, 2005).

The UPR is known to signal through three ER transmembrane sensors: PKR-like ER-regulated kinase (PERK), inositol requiring enzyme1 α (IRE1 α) and activating transcription factor6 (ATF6) (Kim *et al*, 2008; Malhotra *et al*, 2008; Scheuner & Kaufman, 2008). These then activate an adaptive response that results in inhibition of protein translation and increase in transcription of protein-folding chaperones and ER-associated degradation genes (Ozcan *et al*, 2004; van der Kallen *et al*, 2009). ATF6 induces X-box binding protein 1 (XBP1) transcription and IRE1 α upon activation initiates splicing of XBP1 (XBP1s) mRNA which encodes a transcriptional activator that modulates the UPR through regulation of transcription of ER chaperones (Yoshida *et al*, 2001). PERK phosphorylates the eukaryotic translation initiation factor 2 α (eIF2 α) (Harding *et al*, 1999). Phosphor-eIF2 α (p-eIF2 α) then attenuates protein synthesis and reduces ER protein overload (Harding *et al*, 2000a; Harding *et al*, 2000b).

This also results in increased alternative translation of activation transcription factor 4 (ATF4), which induces expression of many genes, including those involved in apoptosis: C/EBP homologous protein (CHOP) (Fawcett *et al*, 1999; Harding *et al*, 2000a; Szegezdi *et al*, 2006; van Huizen *et al*, 2003). Upon activation during UPR, the cytoplasmic domain of ATF6 is cleaved and the cleaved N-terminal fragment translocates to the nucleus and activates the transcription of ER chaperones such as glucose regulated protein (Grp)78/Bip, protein disulfide isomerase (PDI), Ero1-L α and calnexin to augment the ER protein folding capacity (Gregor & Hotamisligil, 2007a; Gregor & Hotamisligil, 2007b; Hotamisligil, 2007; Scheuner & Kaufman, 2008; van der Kallen *et al*, 2009).

Current studies have shown that an enhanced level of the UPR occurs in obese, insulin-resistant human AT (Boden *et al*, 2008; Gregor *et al*, 2009; Sharma *et al*, 2008). ER stress and the UPR are linked to major inflammatory and stress-signalling networks, including the activation of JNK and IKK-NF κ B pathways and the production of reactive oxygen species (ROS) (Hotamisligil, 2006). Intriguingly, these are also the pathways and mechanisms that play a central role in obesity-induced inflammation and metabolic abnormalities (Gregor & Hotamisligil, 2007b). In order to mediate a reduction in increased diabetic risk, previous rodent and human studies examined whether high doses of salicylates can offer any benefits, these studies suggest that salicylates can reduce hyperglycaemia (Baron, 1982). In addition, (Yuan *et al*, 2001) indirectly investigated the potential mechanisms of hyperglycaemic effects. In their study, severely obese rodents treated with salicylates demonstrated reduced signalling through the IKK β pathway and this was accompanied by improved insulin sensitivity *in vivo*.

By inhibiting IKK β activity, salicylates inhibit the activation of nuclear factor- κ B (NF- κ B) via inhibition of phosphorylation and degradation of I κ B α (Kopp & Ghosh, 1994; Pierce *et al*, 1996). This inhibition of NF- κ B may explain some of the clinically documented anti-inflammatory and insulin sensitising effects of salicylates.

Although ER stress, increased AT inflammation and metabolic dysfunction is associated with obesity in rodent models, the importance of ER stress and the potential inducers of ER stress in human adipocytes is not known. Therefore, the objective of the present study was to firstly show the existence of ER stress in human AT and examine changes with and without obesity; secondly, to identify the potential effect of hyperglycaemic like conditions on *in vitro* treated human AbSc adipocytes and finally ascertain the role of the anti-inflammatory agent, sodium salicylate (Sal) on ER stress in cultured primary human adipocytes in the presence and absence of high glucose conditions.

4.2 Research Design and Methods

4.2.1 Subjects

Human AbSc AT was collected from patients (age: 40.8 (mean \pm SD) \pm 5yrs; Lean BMI: $22.04 \pm 2.6 \text{ kg/m}^2$ and obese BMI $30 \pm 3.5 \text{ kg/m}^2$) undergoing elective or liposuction surgery with informed consent obtained in accordance with LREC guidelines and with ethics committee approval. All tissue samples were flash frozen and/or utilised for isolation of stromal fraction, subsequently used for culturing primary human adipocytes as detailed (McTernan *et al*, 2002). In total, 20 human non-diabetic AT samples were analysed, which were sub-divided into: Lean AbSc (n=10) and Obese AbSc (n=10) based on BMI.

4.2.1.1 Cell Culture

In brief, AbSc AT was digested with collagenase (Worthington Biochemical, Reading, USA) as previously described to isolate mature adipocytes and pre-adipocytes (Harte *et al*, 2003). Firstly, stromal fractions of Human AbSc AT (BMI $25.04 \pm 0.6 \text{ kg/m}^2$; n = 3-6) were cultured into tissue culture flasks until confluent and then trypsinised to obtain cells to carry out the study. The preadipocytes from the same passage were grown in 6-well plates to confluence in DMEM/Ham's F-12 phenol-free medium (Invitrogen, Paisley, UK) containing 10% FCS, penicillin (100 U/ml), streptomycin (100 $\mu\text{g/ml}$), and transferrin (5 $\mu\text{g/ml}$).

At confluence, preadipocytes were differentiated in preadipocyte differentiation media (Promocell, Germany) containing biotin (8 µg/ml), insulin (500 ng/ml), Dexamethasone (400 ng/ml Promocell, Germany), IBMX (44 µg/ml Promocell, Germany), L-Thyroxin (9ng/ml Promocell, Germany) and Ciglitazone (3µg/ml Promocell, Germany) for 72 hrs. After this period, the differentiating cells were grown in nutrition media containing DMEM/Ham's F-12, 3% FCS, d-biotin (8 µg/ml), insulin (500 ng/ml) and Dexamethasone (400 ng/ml) until fully differentiated (14-18 days). The viability of adipocytes was assessed using the trypan blue dye exclusion method (Sigma-Aldrich Corp., Poole, UK) (Matthews *et al*, 1985).

4.2.1.2 Treatments

Fully differentiated adipocytes (day 15) were grown in normal DMEM/Ham's F-12 phenol-free medium containing only 2% serum (detoxification media) for 24 hrs to remove effects of growth factors and other components in nutrition media. The treatments were then placed in the fresh detoxification media for 24 hrs. The cells were treated with tunicamycin (tunicamycin, Tun: 750ng/ml); tunicamycin is a well known ER stress inducer and therefore was used as a positive control, glucose (25mM) (Nelson *et al*, 2000; Zhang *et al*, 2009; Zu *et al*, 2008), referred hereafter as high glucose (HG) (Sigma-aldrich, Poole UK) and sodium salicylate (Sal: 20mM; Sigma-aldrich, Poole UK for 24 hrs.

4.2.1.3 Immunoblotting

Cells were washed in PBS and harvested in 250 µl of lysis buffer (20 mM Tris-HCl, pH 7.5; 137 mM NaCl; 1mM EDTA, pH 8; 1% Triton X-100; 10% glycerol; 1.5mM MgCl₂; Sigma-aldrich, Poole UK) containing protease and phosphatase inhibitors (10 mM NaF; 1 mM PMSF; 1 mM sodium metavanadate; 5 µg/ml aprotinin; 10 µg/ml leupeptin; Sigma-aldrich, Poole UK) and stored at -80°C until required. Homogenised human AT was extracted using RIPA buffer method (Harte *et al*, 2003). Total protein was determined by Bradford assay (BioRad, UK). Protein (10-20 µg) from cell lysates were resolved by SDS-PAGE and transferred to an Immobilon-P membrane (Millipore, Hertfordshire, UK) by electroblotting. The membranes were blocked with 0.2% I-BlockTM (Applied Biosystems, Warrington, UK) dissolved in 0.1% Tween20 in PBS and probed with primary and secondary antibodies. Primary antibodies were: phospho and total Akt, phospho and total eIF2 α , Bip/GRP78, Calnexin, Protein disulfide isomerase (PDI), Ero1-L α , phospho-PERK (p-PERK), IRE1 α and β -actin (Cell Signalling Technologies, Massachusettes, USA). Antigen-antibody complexes were visualized using ECL reagents (Amersham, UK). Scanned autoradiographs were semi-quantified using 2D densitometry, GeneTools software (Syngene, Cambridge, UK). The bands were first normalised as a function of the loading control (protein of interest/ β -actin) or total expression of the proteins (for phospho-proteins), then converted to fold change compared with controls.

4.2.1.4 Extraction of RNA and Quantitative RT-PCR

To characterise gene expression, RNA was extracted from adipocytes, according to manufacturers' instructions (RNeasy Lipid Tissue Mini Kit, Qiagen, UK). Following DNase treatment and reverse transcription, mRNA expression levels were determined using an ABI 7500 Real-time PCR Sequence Detection system (Anderson *et al*, 2001). Pre-optimized quantitative primer and probe sequences for genes were utilised (Applera, Cheshire, UK). All reactions were multiplexed with the housekeeping gene 18S, provided as a pre-optimised control probe (Applera, Cheshire, UK), enabling data to be expressed in relation to an internal reference to allow for differences in relative threshold efficiency. Data were obtained as cycle threshold (Ct) values (the cycle number at which logarithmic PCR plots cross a calculated threshold line) and used to determine ΔCt values ($\Delta\text{Ct} = \text{Ct of 18S housekeeping gene} - \text{Ct of gene of interest}$). Measurements were carried out on at least three occasions for each sample. To exclude potential bias due to averaging, all statistics were performed at this stage prior to the data being transformed through the power equation $2^{-\Delta\Delta\text{Ct}}$.

4.2.1.5 Statistical Analysis

Data in the text and figures are presented as mean \pm standard error of the mean (SEM) for at least three independent experiments performed in triplicates to ensure reproducibility. Student's *t* test was used to compare values between two groups unless stated otherwise. *P* values <0.05 were considered to represent statistically significant differences.

4.3 Results

4.3.1 ER Stress Markers are Up-regulated in Obese Human AbSc AT Compared with Lean

Protein expression of the ER stress markers was measured in 4 obese and 4 lean human AbSc AT samples. The p-PERK and IRE1 α proteins, which are regulators of two important ER stress pathways, were increased in obese subjects – although this was only significant for IRE-1 α expression compared with lean (**Figure 4.3.1.1**). ATF6, which regulates the third ER stress pathway, was investigated by examining mRNA expression via real-time PCR, as this proved a more reliable method, with previous studies by (Namba *et al*, 2007) demonstrating that up-regulation of ATF6 mRNA expression is involved in enhancing the ER stress response and is a good marker for the ATF6 ER stress pathway. ATF6 mRNA expression in AbSc AT of 10 lean and 10 obese subjects was significantly higher (8 fold) in obese subjects ($\Delta C_t=07.36\pm1.47$) compared with lean ($\Delta C_t=10.57\pm1.13$) ($p<0.001$) (**Figure 4.3.1.2**). Furthermore, the protein expression of down-stream targets of the three ER stress pathways, *i.e.* the chaperone proteins Grp78/Bip1, Calnexin, PDI and Ero1-L α , were all significantly increased in AbSc AT of obese subjects (**Figure 4.3.1.1**). Protein expression was measured by normalising against the endogenous control, β -actin. The data demonstrate consistent up-regulation of ER stress markers in obese AbSc AT, as previously shown by three different groups (Boden *et al*, 2008; Gregor *et al*, 2009; Sharma *et al*, 2008).

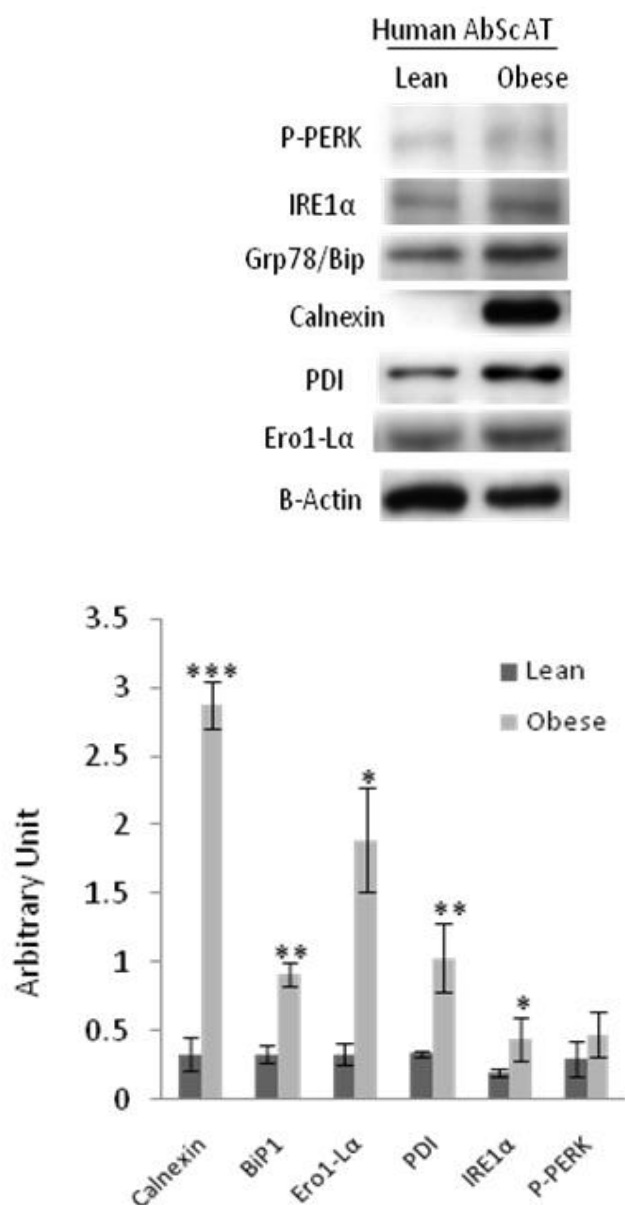


Figure 4.3.1.1 Protein expression levels of ER stress markers: p-PERK, IRE1 α , Grp78/Bip, Calnexin, PDI, Ero1-L α and β -actin (loading control) in lean (n=4) vs. Obese (n=4) human AbSc AT. The protein expression was determined from whole AT lysate by western blot. *p<0.05, **p<0.01 and ***p<0.001 by Student's *t*-test.

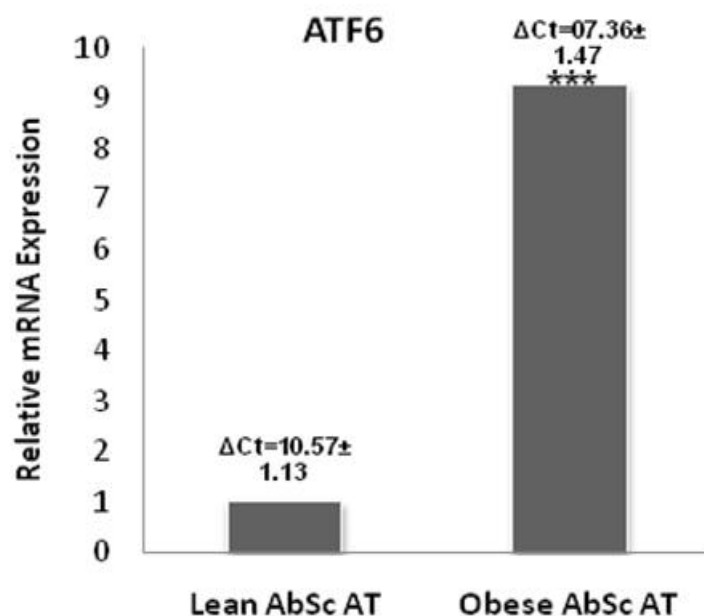
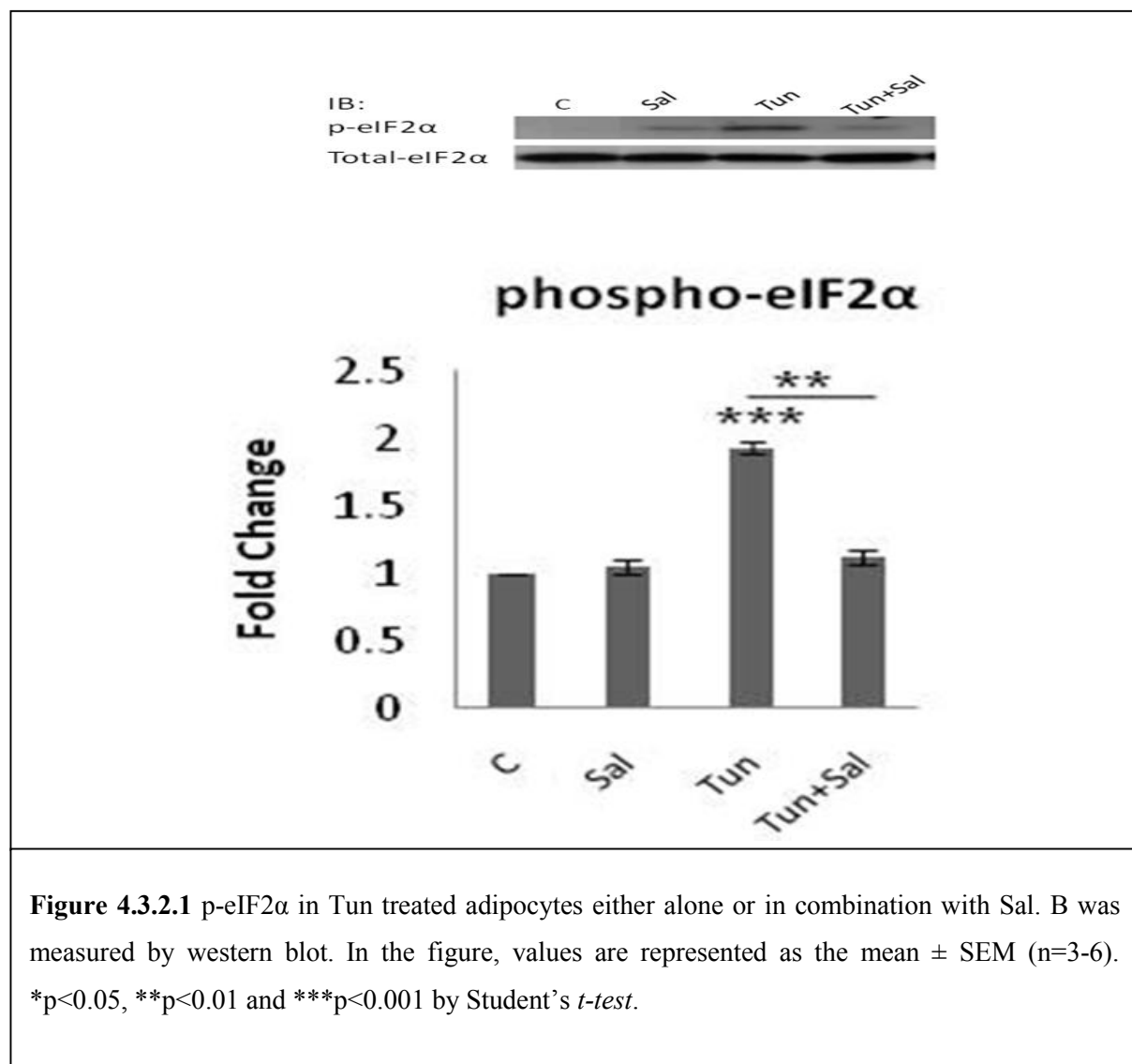


Figure 4.3.1.2 mRNA expression of ATF6 in human AbSc AT, Lean vs Obese: both n=10. mRNA was determined by qRT-PCR. In the figure, values are the mean ± SEM expressed as a relative fold difference to lean AbSc AT, given an arbitrary value of 1. *p<0.05, **p<0.01 and ***p<0.001 by Student's t-test.

4.3.2 Sal Down-regulates PERK and ATF6 Pathway Up-regulated by Tun and HG in Fully Differentiated Primary Human Preadipocytes

Proximal events for the UPR include the activation of ER-resident signaling molecules such as PERK and ATF6 in order to initiate transcriptional and translational programs that alleviate ER stress. To determine the origins of ER stress in human adipocytes, stromal fractions from human AbSc AT were cultured and differentiated into adipocytes (**Figure 4.3.2.1**) and these primary adipocytes were then treated with most probable factors elevated in obesity such as HG for 24 hrs.

Tun which is a known inducer of ER stress was used as a positive control. The protein, p-eIF2 α , is the down-stream-target of p-PERK and was significantly induced by Tun and HG - both of them significantly induced the p-eIF2 α expression compared with cells treated in absence of treatment (control) (**Figure 4.3.2.1 and 4.3.2.2**). We were also interested in looking at the effect of Sal on this pathway. Sal significantly down-regulated the expression of p-eIF2 α in adipocytes treated with Tun and HG in combination with Sal (**Figure 4.3.2.1 and 4.3.2.2**). There was no change in the total eIF2 α expression levels. This provides the first strong evidence that HG activates the PERK pathway, whilst Sal alleviates this activation in human differentiated adipocytes.



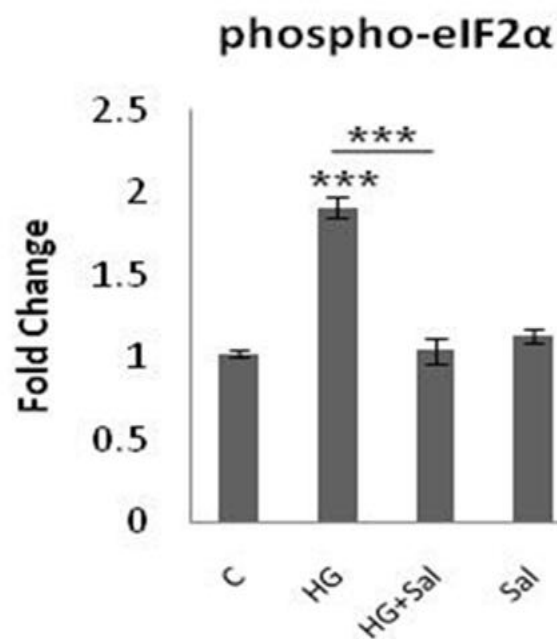
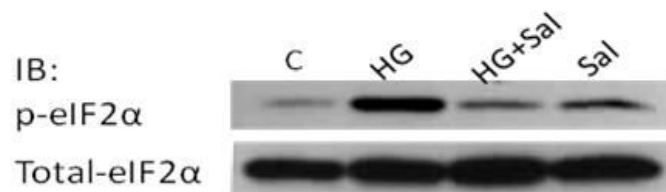
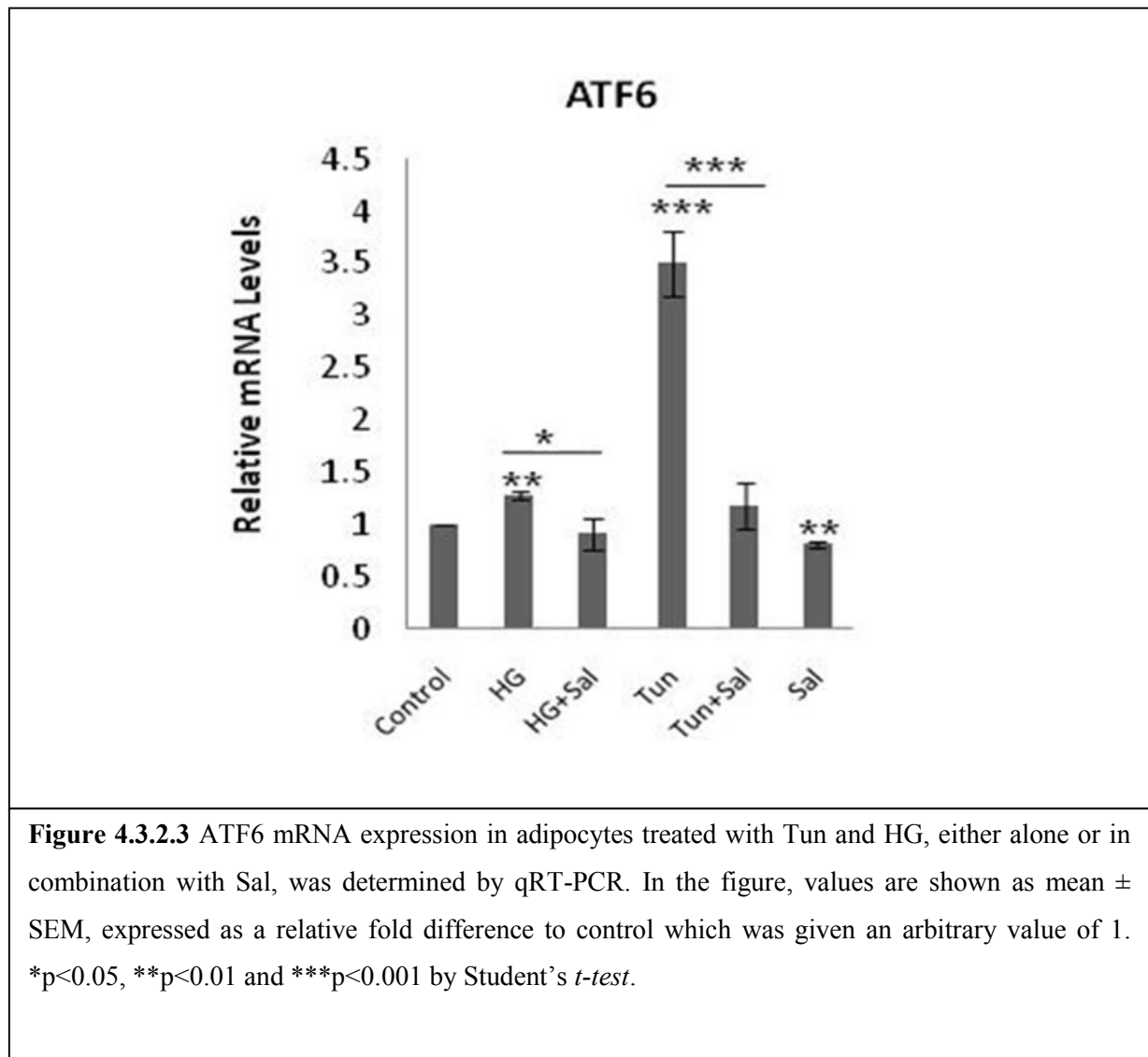


Figure 4.3.2.2 p-eIF2α in HG treated adipocytes, either alone or in combination with Sal, measured by western blot (n=3-6). In the figure, values represent the mean \pm SEM expressed as a relative fold difference to control given an arbitrary values of 1. *p<0.05, **p<0.01 and ***p<0.001 by Student's t-test.

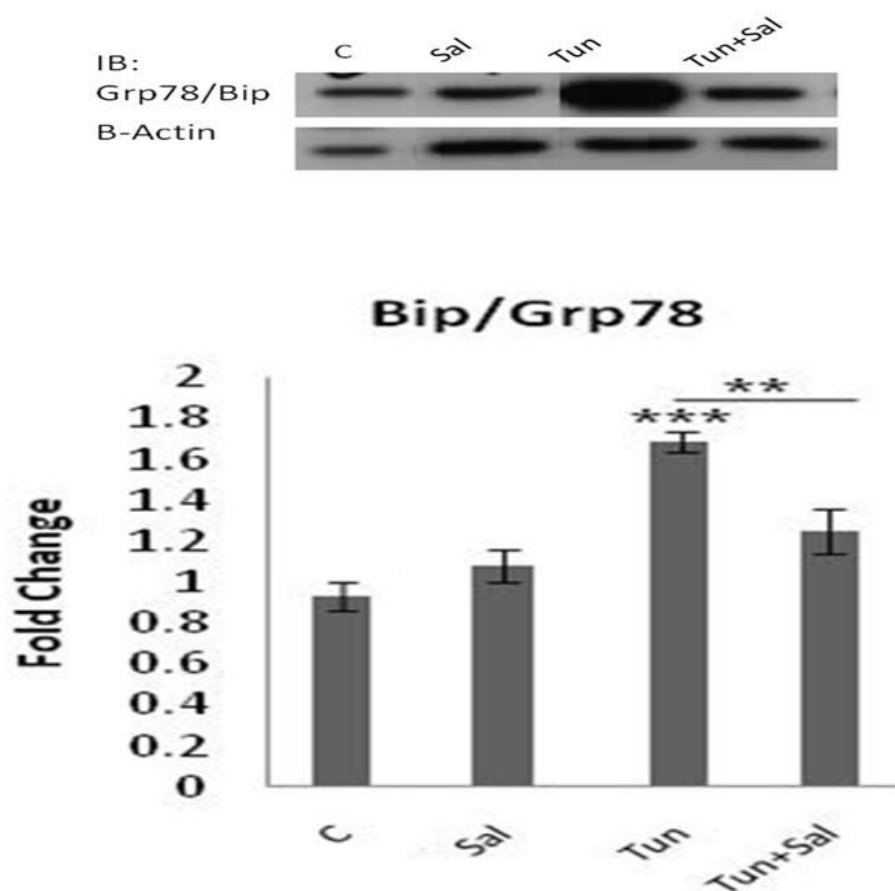
The ATF6 mRNA level was significantly increased in the cells treated with HG and Tun (Figure 4.3.2.3). ATF6 mRNA levels were significantly down-regulated in adipocytes treated with HG and Tun in combination with Sal, and Sal alone (Figure 4.3.2.3).



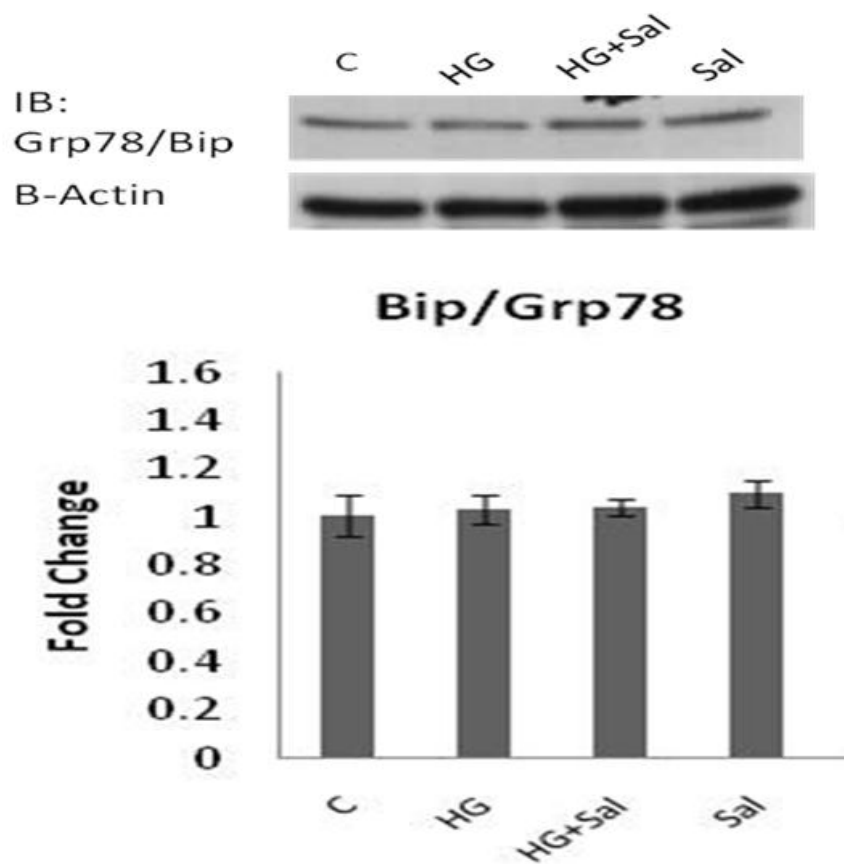
4.3.3 Down-stream Targets of PERK and ATF6 Pathways are Up-regulated by Tun and HG: Sal Alleviates ER Stress Response

Protein chaperones and down-stream targets of PERK and ATF6 pathways either protein or mRNA expression levels were measured in the adipocytes treated with Tun and HG alone or in combination with Sal. ER resident proteins such as Grp78/BiP, calnexin, PDI and Ero1-L α

act as molecular chaperones to promote proper folding and/or prevent aggregation of folding intermediates. UPR is induced when Grp78/Bip expression is induced and the PERK, ATF6 and IRE1 α which are bound to Grp78/Bip, dissociate and are activated (Hotamisligil, 2007). Grp78/Bip was significantly increased in Tun treated cells only (**Figures: 4.3.3.1, 4.3.3.2**). HG did not have any effect on Grp78/Bip expression. Sal significantly down-regulated the expression of Grp78/Bip in Tun treated cells (**Figures: 4.3.3.1, 4.3.3.2**).

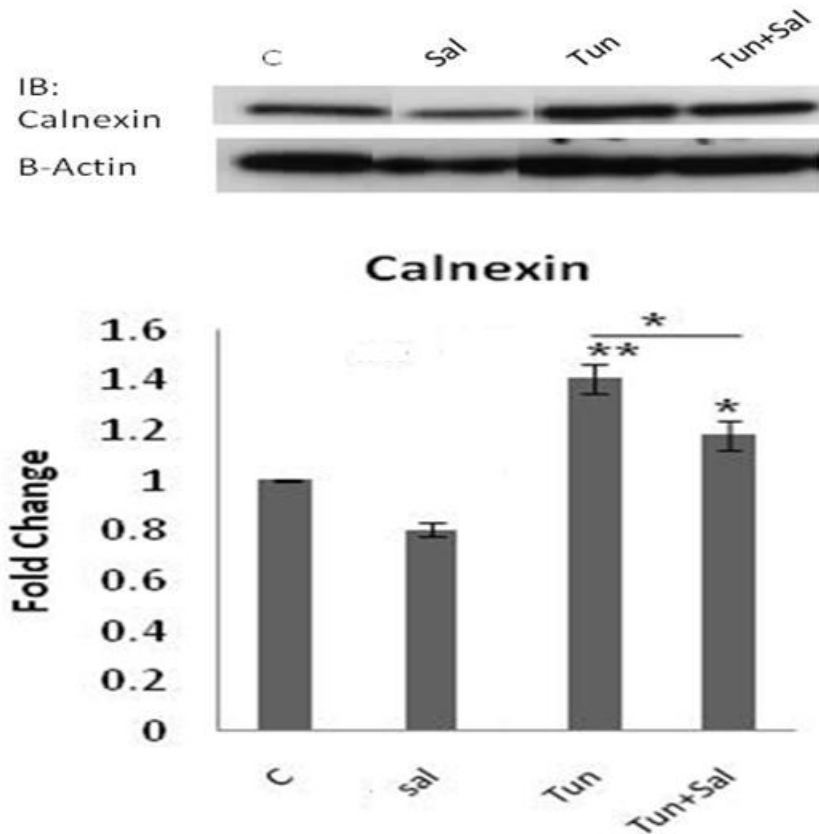


Figures: 4.3.3.1 Protein expression levels of Grp78/Bip in Tun treated adipocytes, either alone or in combination with Sal, measured by western blot (n=3-6). In the figure, values show the mean \pm SEM, expressed as a relative fold difference to control which was given an arbitrary value of 1. *p<0.05, **p<0.01 and ***p<0.001 by Student's t-test.

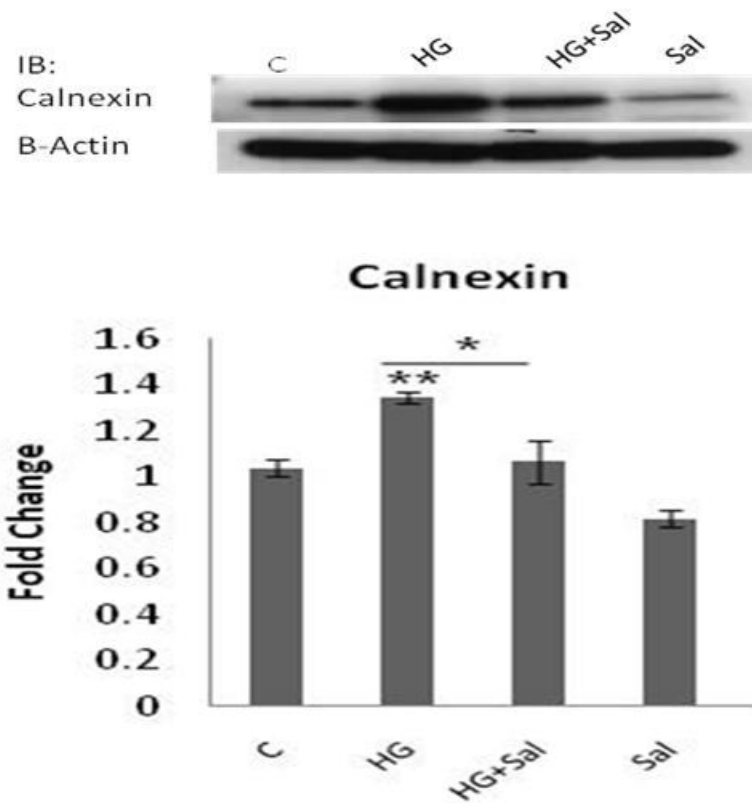


Figures: 4.3.3.2 Protein expression levels of Grp78/Bip in HG treated adipocytes, either alone or in combination with Sal, by western blot (n=3-6). In the figure, values show the mean \pm SEM, expressed as a relative fold difference to control and given an arbitrary value of 1. No significant differences were noted compared with control.

Another protein chaperone, Calnexin, was significantly up-regulated by Tun and HG but this effect was significantly down-regulated by Sal (**Figures: 4.3.3.3, 4.3.3.4**). Sal alone also significantly suppressed the Calnexin expression compared with control.

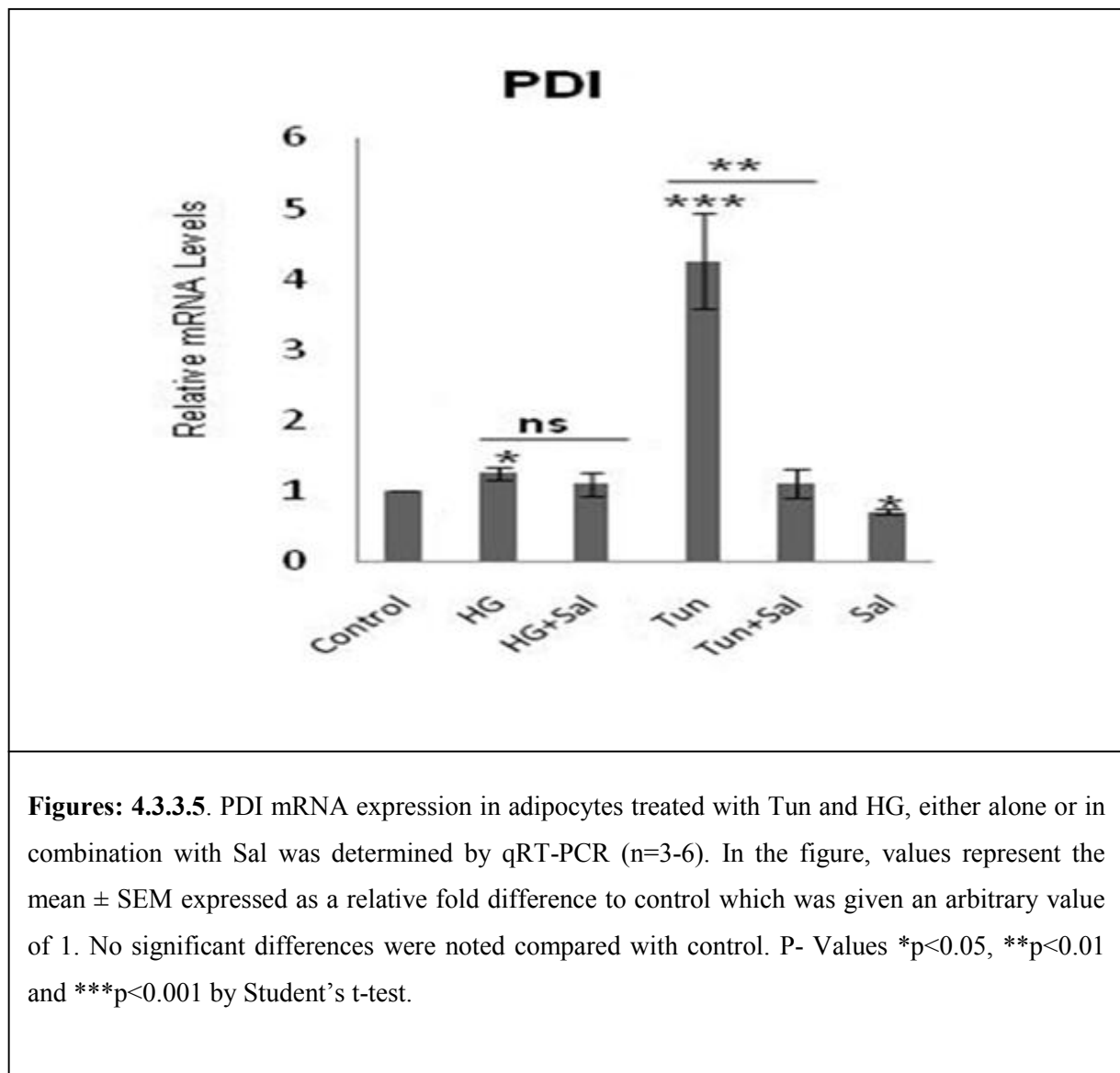


Figures: 4.3.3.3 Protein expression levels of Calnexin in Tun treated adipocytes, alone and in combination with Sal, by western blot (n=3-6). In the figure, values represent the mean \pm SEM expressed as a relative fold difference to control given an arbitrary value of 1. No significant differences were noted compared with control. P- Values *p<0.05, **p<0.01 and ***p<0.001 by Student's t-test.



Figures: 4.3.3.4 Protein expression levels of Calnexin in HG treated adipocytes, alone and in combination with Sal, by western blot (n=3-6). In the figure, values show the mean \pm SEM expressed as a relative fold difference to control and given an arbitrary value of 1. No significant differences were noted compared with control. P- Values * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ by Student's t-test.

The mRNA expression of the protein chaperones, PDI and Ero1-L α , were significantly increased in Tun and HG treated adipocytes (**Figures: 4.3.3.5 and 4.3.3.6**). Sal, again, significantly down-regulated the expression of these chaperones in the adipocytes treated in combination with Sal. Only PDI wasn't significantly suppressed by Sal in HG treated cells. However, Sal alone significantly down-regulated PDI mRNA expression levels (Figure 4.3.3.5).



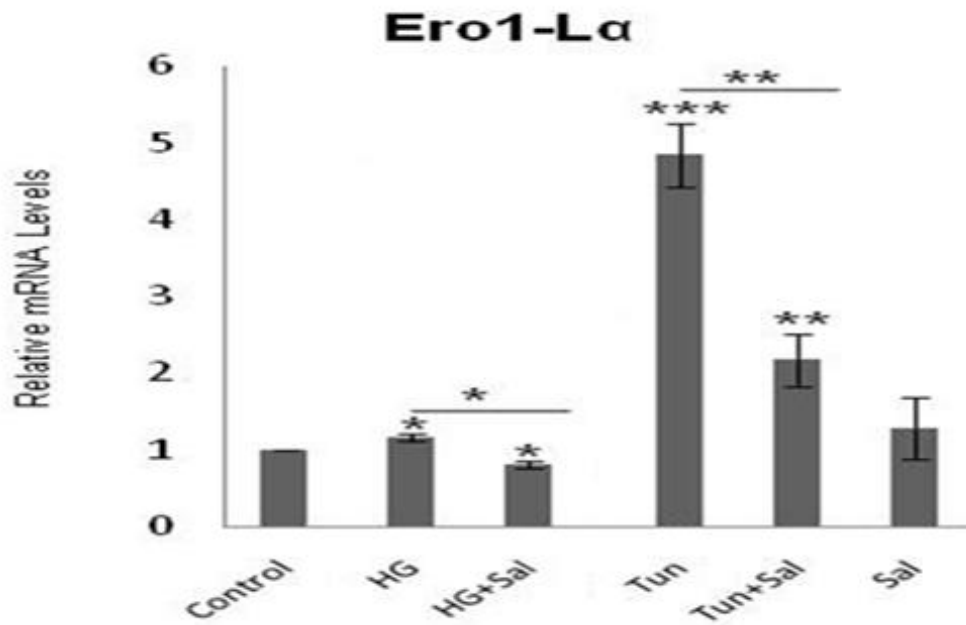


Figure 4.3.3.6 Ero1-L α mRNA expression in adipocytes treated with Tun and HG, either alone or in combination with Sal, was determined by qRT-PCR (n=3-6). In the figure, values show the mean \pm SEM expressed as a relative fold difference to control and given an arbitrary value of 1. No significant differences were noted compared with control. P- Values *p<0.05, **p<0.01 and ***p<0.001 by Student's t-test.

Signalling through PERK, ATF6 and IRE1 α can induce pro-apoptotic signals during extreme ER stress by activating down-stream targets such as CHOP. Therefore, we examined the mRNA expression of ER stress-induced apoptotic transcription factor CHOP in Tun and HG treated primary adipocytes. Both treatments significantly increased CHOP expression (at least 2-fold) (**Figure 4.3.3.7**). Interestingly, Sal significantly down-regulated CHOP expression induced by Tun and HG (**Figure 4.3.3.7**).

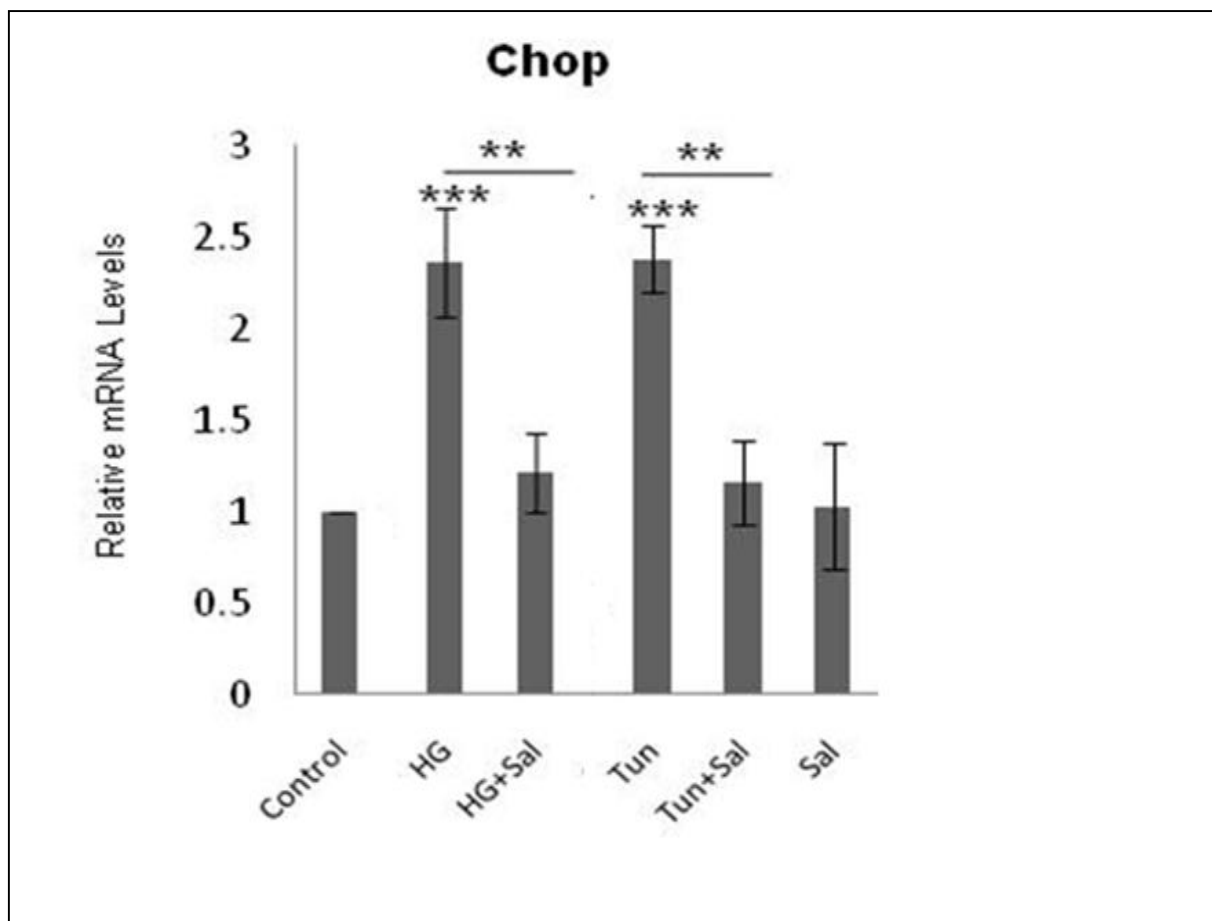
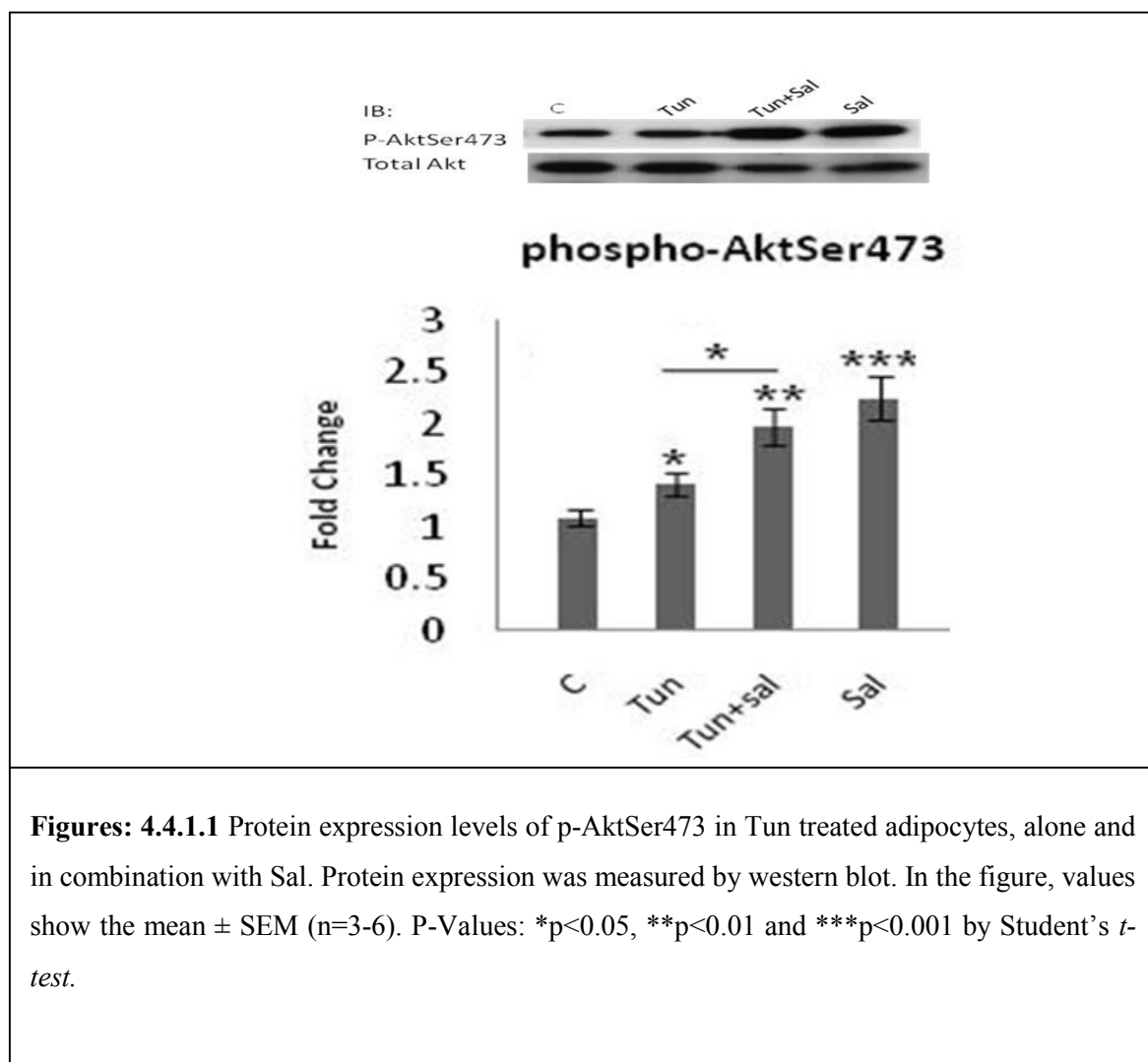
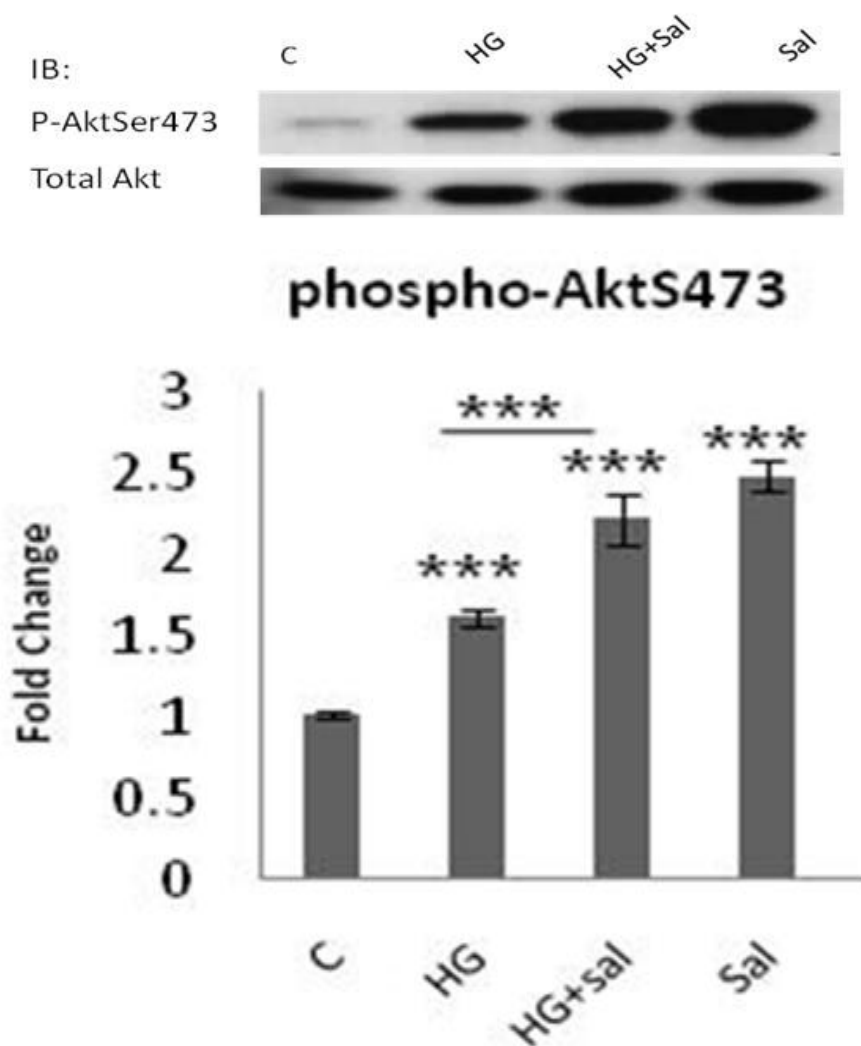


Figure 4.3.3.7 CHOP mRNA expression in adipocytes treated with Tun and HG, either alone or in combination with Sal, was determined by qRT-PCR (n=3-6). In the figure, values represent the mean \pm SEM expressed as a relative fold difference to control and given an arbitrary value of 1. No significant differences were noted compared with control. P- Values * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ by Student's t-test.

4.4.1 Activation of AktSer473

Hu *et al.* (Hu *et al.*, 2004) have reported transient activation of Akt in ER-induced apoptosis and p-Akt (Ser473) is an important member of the insulin signalling pathway, as it induces glucose transport and overall insulin sensitivity of the cell. Therefore, Akt activation was examined by measuring p-AktSer473 levels in the adipocytes treated with Tun and HG, and in combination with Sal. Interestingly, Tun and HG significantly induced p-AktSer473 levels compared with controls. There was further significant induction by Sal in adipocytes treated with Tun and HG (Figures: 4.4.1.1 and 4.4.1.2). Sal, alone, significantly increased p-AktSer473 levels (Figures: 4.4.1.1 and 4.4.1.2).





Figures: 4.4.1.2 Protein expression levels of p-AktSer473 in HG treated adipocytes, alone and in combination with Sal. Protein expression was measured by western blot. In the figure, values represent the mean \pm SEM (n=3-6). P-Values: * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ by Student's *t*-test.

4.5 DISCUSSION

The AT plays a central role in the regulation of metabolic homeostasis. The AT in obesity is challenged by many insults, such as surplus energy, inflammation, insulin resistance and considerable stress to various organelles. ER is one such organelle which shows significant signs of stress and has been casually linked as one of the contributing factors for increased inflammation and insulin resistance. The results of the present study demonstrate that firstly, there is increased ER stress in obese AbSc AT; secondly, that one factor inducing this response could be hyperglycaemia and thirdly, that this stress response is alleviated by salicylates and could contribute to increased insulin sensitivity in adipocytes. Hyperglycaemia has been shown to be present in obese subjects and has been linked to increased inflammation and insulin resistance (Belfiore *et al*, 2001) and, therefore, was investigated as a potential factor for the induction of ER stress in human adipocytes. Tun is a well known ER stress inducer and therefore was used as a positive control.

ER stress protein markers were significantly up-regulated in obese human AbSc AT compared to AbSc AT from lean subjects. The IRE1 α and ATF6 expression levels were significantly increased in obese AbSc AT. The p-PERK was also increased but this was not significant. This observation confirms the three previous studies carried out on human AT (Boden *et al*, 2008; Gregor *et al*, 2009; Sharma *et al*, 2008). PERK, ATF6 and IRE1 α play a central role in UPR signalling. Upon activation, these factors induce the expression of protein chaperones for the proper folding of the protein and protein complexes (Eizirik *et al*, 2008; Gething & Sambrook, 1992).

The protein chaperones Grp78/Bip, Calnexin, Ero1-L α and PDI were all significantly up-regulated in obese human AbSc AT compared with lean AbSc AT. Increased expression of PDI and Calnexin confirms the earlier observation made by Boden and colleagues (Boden *et al*, 2008). To identify the factors responsible for inducing ER stress in human AT, stromal fractions isolated from human AT were cultured and then differentiated. The differentiated adipocytes were then treated with Tun and HG with and without salicylate. Our data clearly demonstrates that Tun and HG activate the PERK pathway. This activation was measured by looking at the expression levels of p-eIF2 α , a down-stream target of PERK, which was significantly increased in Tun and HG treated adipocytes. PERK is activated in response to accumulation of misfolded proteins in the ER, reducing the rate of protein synthesis through eIF2 α phosphorylation at ser51 to assure proper protein folding (Harding *et al*, 2000a; Harding *et al*, 2000b), whilst also inducing the transcription of protein chaperones. P-eIF2 α activation was totally eliminated when the above treatment was given in combination with salicylate. The observation in this study appears to contradict previous work in promonocytic cell line THP-1 where salicylate and aspirin have been shown to induce eIF2 α phosphorylation and hence protein synthesis attenuation (Silva *et al*, 2007). One explanation for the apparent difference could be that, in that study the cells were exposed to salicylates or aspirin for a very short time, a maximum of 3 hrs for salicylate and 6 hrs for aspirin while our observations are based on 24 hr exposure. Alternatively, it could be a cell specific response. The current observation within this study is further supported by the significant increase in expression of protein chaperones, specifically CHOP by Tun and HG, the induction of which was significantly down-regulated by Sal.

Previous findings have demonstrated that to up-regulate CHOP transcription the PERK-eIF2 α -ATF4 branch of the UPR is essential (Szegezdi *et al*, 2006).

Modification of ATF6 protein is important for the ER stress response. ER stressors stimulate the cleavage of ATF6 by Site-1 protease (S1P) and Site-2 protease (S2P) into p50-ATF6, which acts as a transcription factor. Namba and co-workers have shown that all the ER stressors they tested (such as thapsigargin and Tun) up-regulated ATF6 mRNA expression with the cells over-expressing ATF6 mRNA showing an enhanced ER stress response (Namba *et al*, 2007). Indeed the adipocytes treated with Tun in this study showed highly significant induction of ATF6 mRNA expression compared with the controls. Therefore, up-regulation of ATF6 mRNA expression could be used as an indicator of activation of the ATF6 pathway. HG also induced ATF6 mRNA expression significantly. Salicylate significantly down-regulated the ATF6 mRNA expression induced by Tun and HG. Induction of the ATF6 pathway is supported by the increase in expression of protein chaperones up-regulated by ATF6. The protein expression of the chaperones, Calnexin, PDI, Ero1- α and CHOP, was up-regulated in adipocytes treated with Tun and HG. Sal again contributed to the attenuation of the ER stress by down-regulating both the PERK and ATF6 pathways. The most interesting observation involved Grp78/Bip expression and its effect on hyperglycaemic conditions. The Grp78/Bip expression was significantly increased only by Tun, which was in turn down-regulated by Sal. Despite considerable activation of the UPR, HG did not induce Grp78/Bip expression. Studies by Zhang have made the same observations in INS-1 pancreatic β -cells treated with HG (30mM).

The reason for this is, as yet, undetermined but would be of considerable interest as Bertolotti and co-workers (Bertolotti *et al*, 2000) have shown that PERK is found complexed with Grp78/Bip in cells in conditions where the cell has no apparent ER related stress. In order to activate eIF2 α , it must dissociate from Grp78 /Bip under UPR condition and our results clearly demonstrate that the p-eIF2 α and its down-stream target CHOP are activated by HG. It is quite possible that another mechanism exists for PERK activation under hyperglycaemic conditions.

The present study also investigated whether these UPR inducing factors would have an effect on p-AktSer473 levels. Interestingly, p-AktSer473 was significantly induced by Tun and HG. This is not a unique observation, as Hu *et al*. (Hu *et al*, 2004) also reported transient activation of Akt during ER stress, induced by thapsigargin and Tun in MCF-7 cells. They have also demonstrated that blocking Akt activity sensitised MCF-7 cells to ER stress-induced apoptosis, suggesting that Akt activation is a pro-survival pathway, activated during ER stress. Additionally studies by Ho and co-workers (Ho *et al*, 2006) have shown that Akt is activated under hyperglycaemic conditions (33mM Glucose) in human umbilical vein endothelial cells (HUVECs) within 24 hrs. The adipocytes treated with Sal alone, or in combination with Tun or HG, induced p-AktSer473 significantly, at least two fold higher than HG alone. This could be because of the anti-inflammatory and insulin sensitising effect of salicylates where it inhibits the activation of nuclear factor- κ B (NF- κ B) via inhibition of phosphorylation and subsequent degradation of I κ B α (Kopp & Ghosh, 1994; Pierce *et al*, 1996). Salicylate is an interesting molecule and in our study we have demonstrated that it successfully alleviates ER stress induced by Tun and HG.

From this study we could also deduce that it has attenuating effects on at least two of the three ER stress pathways, namely, the PERK and the ATF6 pathways. The current study did not investigate the IRE1 α pathway but it remains the objective of future studies. The mechanism by which salicylate ameliorates ER stress is unknown. One possible mechanism could be the one demonstrated by Yuan *et al.* (Yuan *et al.*, 2001) in liver and skeletal muscle tissues of rodents. They and others have shown that salicylates can inhibit the activation of NF- κ B by preventing the phosphorylation and subsequent degradation of I κ B α by down-regulating I κ B kinase β (IKK β) (Yin *et al.*, 1998; Yuan *et al.*, 2001). This mechanism might only be accurate if the ER stress induced by HG was the result of increased inflammation.

In conclusion this chapter has addressed two main issues: 1) what can induce ER stress in human adipocytes and 2) whether salicylate can alleviate the ER stress induced by these factors. We have clearly demonstrated that HG induced significant ER stress in primary human adipocytes, specifically the PERK and ATF6 pathways, and salicylate fully alleviates this stress. In addition, salicylate also induces insulin sensitivity by activating Akt. Future studies will examine the impact of hyperlipidaemia to examine whether this may cause enhanced ER stress beyond that already identified for induced hyperglycaemic conditions on adipose cells, and the impact again of salicylates on this pathway.

Chapter 5

The Effect of Hyperlipidaemia Mediating ER Stress in Human Differentiated Abdominal Subcutaneous Predipocytes

5.1 Introduction

In human studies dietary cholesterol, trans-fatty acids (TFAs) and saturated fatty acids (SFA) increase the levels of atherogenic lipoproteins. As such, current recommendations stress the need to decrease their intake to reduce coronary heart disease (CHD) (Kris-Etherton *et al.*, 2001). However, the best replacement for the calories supplied by dietary SFA is a matter of controversy and the effects they cause are not clearly defined.

Current epidemiological studies examining diet, CHD and metabolic risk have examined dietary effects based on circulating lipoproteins (Brown *et al.*, 2007; Hu *et al.*, 1997; Mozaffarian and Clarke, 2009; Zock *et al.*, 1997). Cross-sectional data suggest that SFA adversely affects vascular function whereas polyunsaturated fat (PUFA; mainly linoleic acid and omega-3 fatty acids (18: 2n-6) n-3 PUFA) appear beneficial (Hall, 2009). Dietary fats, represents an independent risk factor for CVD and previous studies have shown eicosapentaenoic acid (EPA) and DHA (Docosahexaenoic acid) to reduce blood pressure, improve arterial compliance in subjects with T2DM and dyslipidaemia, and augment endothelium-dependent vasodilatation (Jakobsen *et al.*, 2009). Further meta-analysis comparing habitual TFA consumption with CHD outcomes in prospective cohort studies identified that each 2% energy replacement of TFAs with SFAs, mono-unsaturated fatty acids (MUFAs) or PUFAs would lower CHD risk by 17% (SFA; 95% confidence interval (CI)=7-25%), 21% (MUFAs 95% CI=12-30%) or 24% (PUFAs 95% CI=15-33%), respectively (Brown *et al.*, 2007; Jakobsen *et al.*, 2009; Mozaffarian and Clarke, 2009). However these studies merely provide correlation data and not cause-and-effect relationship, nor do such studies help us distinguish between the diets, as many logistical issues prevent this insight such questions and answers remain challenging (Merkel *et al.*, 2001). In addition, it would

seem necessary to address how acute are the post-prandial effects of diet (high SFA, high PUFA, high CHO, HG or fibre rich meal) on systemic inflammation as well as the intracellular response to such an insult (Jakobsen *et al.*, 2009; Kelley *et al.*, 2009).

It is already well established that a prominent feature of the MS is hypertriglyceridaemia, which frequently accompanies a more general dyslipidaemia. Hypertriglyceridaemia has direct effects on the endothelium, but atherosclerotic plaque initiation and progression are also highly related to increased concentrations of small dense LDL, low plasma concentrations of HDL-cholesterol and the increased inflammatory environment (Esteve *et al.*, 2005). Obesity, coupled to the resistance of AT to the antilipolytic effects of insulin, results in a chronic elevation of circulating non-esterified fatty acids (Frayn and Coppack, 1992). This has many important effects including (i) increased TG secretion by the liver, (ii) production of intracellular intermediates (*e.g.* in myocytes, endothelial cells) that interfere with insulin signalling (ceramide, diglycerides), inhibit nitric oxide formation, and promote inflammation, (iii) increased basal insulin secretion and decreased glucose sensitivity of the pancreatic beta-cell and (iv) ectopic accumulation of TG in non-AT cell types *e.g.* myocytes, hepatocytes and pancreatic beta-cells. All these changes lead to increased diabetic risk coupled with atherosclerosis and CVD which are evident within the pre-diabetic period, *i.e.* well before the pancreatic beta-cells have lost the ability to secrete increasing amounts of insulin that compensates for the increased insulin resistance. The adipokines leptin and adiponectin, the concentrations of which vary reciprocally as obesity develops, both stimulate fatty acid oxidation in ectopic tissues, thus protecting them from the effects of excessive delivery of fatty acid moieties (either as unesterified fatty acids or as TG within TG-rich lipoproteins) (Sharma and Chetty, 2005; Valle *et al.*, 2005). In addition, leptin promotes intracellular TG lipolysis in these tissues. As such adipokine secretion, which is increased in insulin resistant conditions, has reciprocal effects on hepatic

glucose production, and thus poor glycaemic control, which indirectly contributes to dyslipidaemia (Valsamakis *et al.*, 2004).

The aims of this chapter were to examine the potential effects of hyperlipidaemic like conditions on *in vitro* treated human AbSc adipocytes and ascertain the role of the anti-inflammatory agent, sodium salicylate (Sal) on ER stress in cultured primary human adipocytes in the presence and absence of hyperlipidaemic conditions; finally to assess the comparative effects on adipokine release in treatments.

5.2 Research Design and Methods

5.2.1 Subjects. Human AbSc AT was collected from patients undergoing elective or liposuction surgery with informed consent obtained in accordance with LREC guidelines and with ethics committee approval. All tissue samples were flash frozen and/or utilised for isolation of stromal fraction, subsequently used for culturing primary human adipocytes as detailed (McTernan *et al.*, 2002).

5.2.2 Cell Culture

AbdSc AT was digested with collagenase (Worthington Biochemical, Reading, USA) as previously described to isolate mature adipocytes and pre-adipocytes (Harte *et al.*, 2003). Firstly, stromal fractions of Human AbSc AT (BMI $25.04 \pm 0.6 \text{ kg/m}^2$; n = 3-6) were cultured into tissue culture flasks until confluent and then trypsinised to obtain cells to carry out the study. The preadipocytes from the same passage were grown in 6-well plates to confluence in DMEM/Ham's F-12 phenol-free medium (Invitrogen, Paisley, UK) containing 10% FCS, penicillin (100 U/ml), streptomycin (100 µg/ml), and transferrin (5 µg/ml). At confluence, preadipocytes were differentiated in preadipocyte differentiation media (Promocell, Germany) containing biotin (8 µg/ml), insulin (500 ng/ml), Dexamethasone (400 ng/ml), IBMX (44 µg/ml), L-Thyroxine (9 ng/ml) and Ciglitazone (3 µg/ml) for 72 hrs. After this period, the differentiating cells were grown in nutrition media containing DMEM/Ham's F-12, 3% FCS, d-biotin (8 µg/ml), insulin (500 ng/ml) and Dexamethasone (400 ng/ml) until fully differentiated (14-18 days). The viability of adipocytes was assessed using the trypan blue dye exclusion method (Sigma-Aldrich Corp., Poole, UK) (Matthews *et al.*, 1985).

5.2.3 Treatments

Fully differentiated adipocytes (day 15) were grown in normal DMEM/Ham's F-12 phenol-free medium containing only 2% serum (detoxification media) for 24 hrs to remove effects of growth factors and other components in nutrition media. The treatments were then placed in the fresh detoxification media for 24 hrs. The cells were treated with Tun (750ng/ml); SFA (2mM), glucose (25mM, Sigma-aldrich, Poole, UK) and sodium salicylate (Sal: 20mM; Sigma-aldrich, Poole, UK), 4-Phenyl butyric acid (PBA: 20mM; Sigma-aldrich, Poole, UK) and Taurine-conjugated ursodeoxycholic acid (TUDCA:500 µg/ml, Sigma-aldrich, Poole UK) for 24 hrs. SFA was prepared as 40mM stocks by dissolving stearic palmitic acid mixture (referred to as SFA (Fluka, UK) in absolute ethanol and then lyophilising it. The lyophilised SFA was re-constituted in 1 ml 3% BSA (Free-fatty acid free) in Geys buffer by vortexing and sonicating. The dissolving buffer without SFA was used as control whenever SFA was used as treatment. All the data shown in this study is from 24 hr treatments, only.

5.2.4 Quantitative Measurement of Secreted Adipokines Concentration From Treated Differentiated Preadipocytes

Conditioned media from control cultured differentiated adipocytes and from treated differentiated adipocytes, were assayed for leptin, and IL-6 using human ELISA kits (leptin, IL-6, Millipore, Cheshire, UK). Conditioned media were analysed using a solid phase enzyme-linked immunosorbant assay, the leptin ELISA had an assay limit of 0.5 ng/ml, intra assay coefficient of variance (CV) of 2.6-4.6% and inter-assay variability was 1.4-4.9%; IL-6 ELISA had an assay limit of 0.3 pg/ml, intra assay coefficient of variance (CV) of 4.5-6.9% and inter-assay variability was 6.2-8.0%. Methodology for the full protocol of a standard ELISA is given in the materials and methods.

5.2.5 Immunoblotting

As outlined previously, cells were washed in PBS and harvested in 250 µl of lysis buffer (20 mM Tris-HCl, pH 7.5; 137 mM NaCl; 1mM EGTA, pH 8; 1% Triton X-100; 10% glycerol; 1.5mM MgCl₂) containing protease and phosphatase inhibitors (10 mM NaF; 1 mM PMSF; 1 mM sodium metavanadate; 5 µg/ml aprotinin; 10 µg/ml leupeptin) and stored at -80°C until required (Harte *et al.*, 2003). Protein (10-20µg) from cell lysates were resolved by SDS-PAGE and transferred to an Immobilon-P membrane (Millipore, Cheshire, UK) by electroblotting. The membranes were blocked with 0.2% I-BlockTM (Applied Biosystems, Warrington, UK); 0.1% Tween20 in PBS and probed with primary and secondary antibodies. Primary antibodies were: phospho and total Akt, phospho and total eIF2 α , Bip/GRP78, Calnexin, Protein disulfide isomerase (PDI), Ero1-L α , phospho-PERK (p-PERK), IRE1 α and β -actin (Cell Signalling Technologies, Massachusettes, USA). Antigen-antibody complexes were visualised using ECL reagents (Amersham, UK). Scanned autoradiographs were semi-quantified using 2D densitometry software (GeneTools, Syngene, UK). The bands were first normalised as a function of the loading control (protein of interest/ β -actin) or total expression of the proteins (for phosphor proteins), then converted to fold change compared with controls.

5.2.6 Extraction of RNA and Quantitative RT-PCR

To characterise gene expression, RNA was extracted from differentiated adipocytes, according to manufacturers' instructions (RNeasy Lipid Tissue Mini Kit, Qiagen, UK). Following DNase treatment and reverse transcription, mRNA expression levels were determined using an ABI 7500 Real-time PCR Sequence Detection system (Anderson *et al.*, 2001). Pre-optimised quantitative primer and probe sequences for genes were utilised (Applera, Cheshire, UK). All reactions were multiplexed with the housekeeping gene 18S,

provided as a pre-optimised control probe (Applera, Cheshire, UK), enabling data to be expressed in relation to an internal reference to allow for differences in relative threshold efficiency. Data were obtained as cycle threshold (Ct) values (the cycle number at which logarithmic PCR plots cross a calculated threshold line) and used to determine ΔC_t values ($\Delta C_t = C_t$ of 18S housekeeping gene subtracted from C_t of gene of interest). Measurements were carried out on at least three occasions for each sample. To exclude potential bias due to averaging, data have been transformed through the Power equation $2^{-\Delta \Delta C_t}$, all statistics were performed at this stage.

5.2.7 Statistical Analysis

Data in the text and figures are presented as mean \pm standard error of the mean (SEM) for at least three independent experiments performed in triplicates to ensure reproducibility. Students' *t* test was used to compare values between two groups unless stated otherwise. *P* values <0.05 were considered to represent statistically significant differences.

5.3 RESULTS

5.3.1 PERK and ATF6 Pathways Altered by SFA in Fully Differentiated Primary Human Preadipocytes

The protein, p-eIF2 α , is the down-stream-target of p-PERK and was significantly induced by Tun and SFA - both of which significantly up-regulated the p-eIF2 α expression compared with cells treated just with the control (**Figures. 5.3.1.1 & 5.3.1.2**). In addition, the anti-inflammatory agent Sal led to a significantly down-regulated expression of p-eIF2 α in adipocytes treated with Tun and SFA in combination with Sal (**Figures. 5.3.1.1 & 5.3.1.2**), whilst there was no change in the total eIF2 α expression levels.

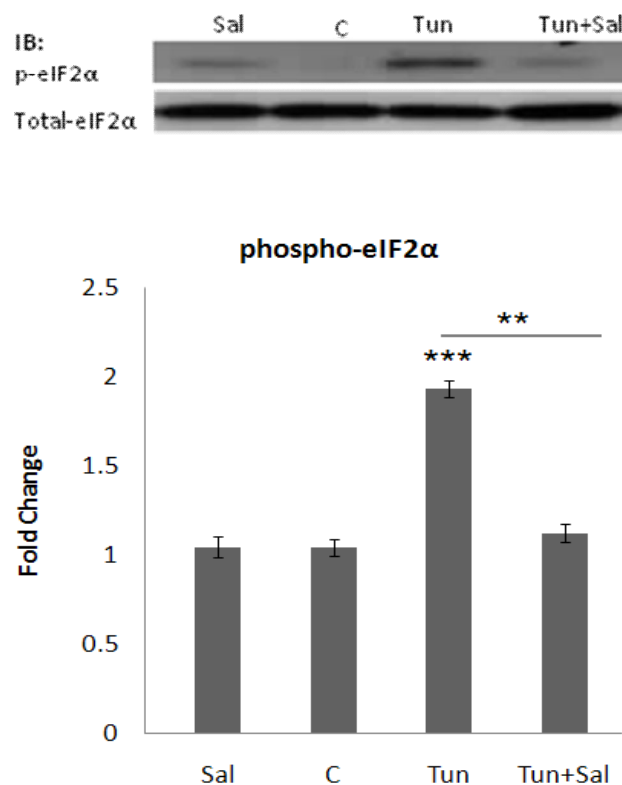


Figure: 5.3.1.1 p-eIF2 α protein expression in Tun treated adipocytes, either alone or in combination with Sal, as measured by Western blot (n=3-6). In the figure, values are presented as the mean \pm SEM expressed as a relative fold difference to control, which was given an arbitrary value of 1. P-Values *p<0.05, **p<0.01 and ***p<0.001 by Student's t-test.

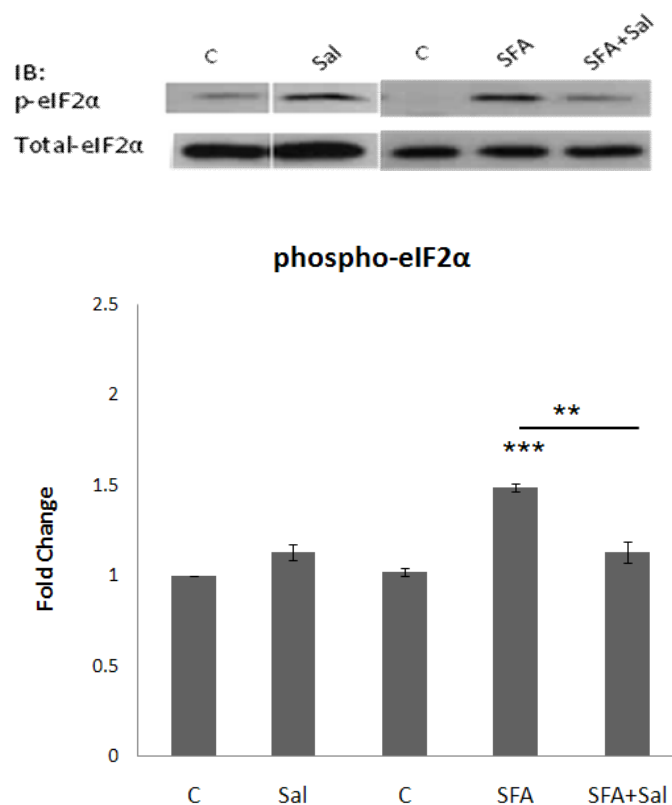


Figure 5.3.1.2 p-eIF2 α protein expression in SFA treated adipocytes, either alone or in combination with Sal, as measured by Western blot (n=3-6). In the figure, values are presented as the mean \pm SEM expressed as a relative fold difference to control, which was given an arbitrary value of 1. P-Values *p<0.05, **p<0.01 and ***p<0.001 by Student's t-test

The ATF6 mRNA level was significantly increased in the cells treated with Tun but was not significant in SFA treated cells (**Figure 5.3.1.3**). ATF6 mRNA levels were significantly down-regulated in adipocytes treated with Tun in combination with Sal and Sal alone (**Figure**

5.3.1.3). Sal also down-regulated ATF6 mRNA expression in SFA treated cells, although this was not significant (**Figure 5.3.1.3**).

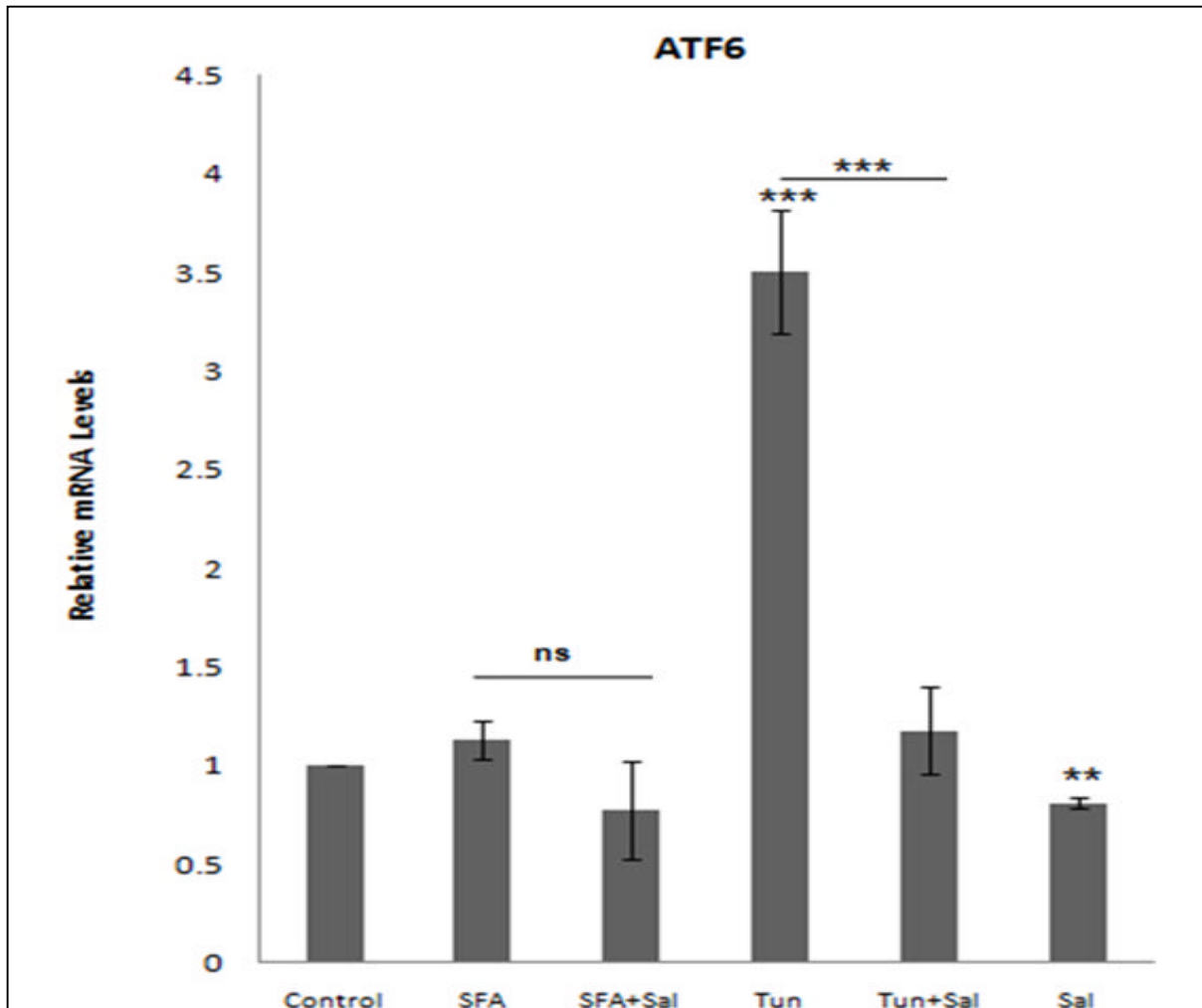
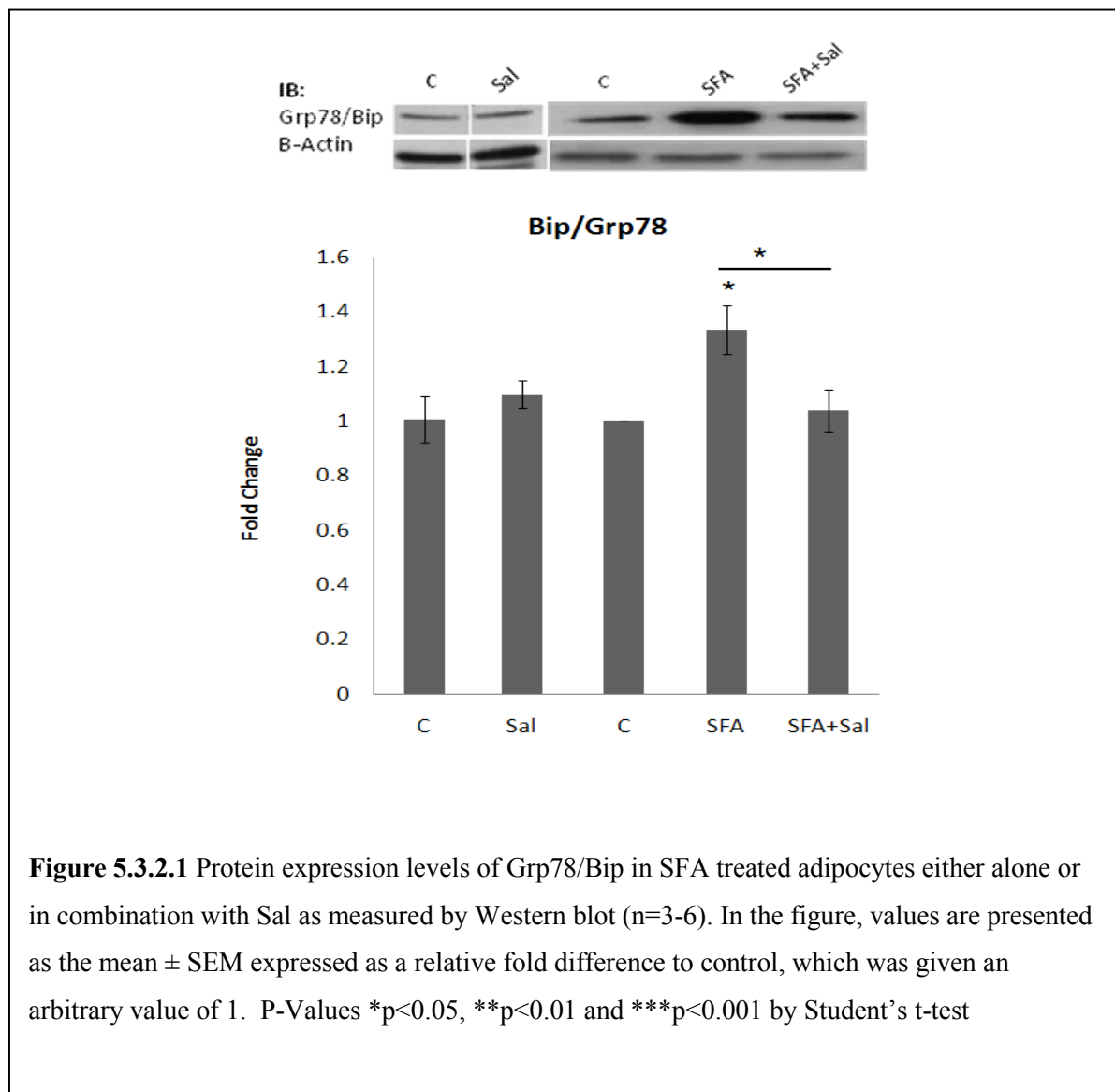


Figure 5.3.1.3 ATF6 mRNA expression in adipocytes treated with Tun and SFA, either alone or in combination with Sal. The mRNA was determined by qRT-PCR (n=6). In the figure, values are presented as the mean \pm SEM expressed as a relative fold difference to control, which was given an arbitrary value of 1. No significant differences were noted compared with control. P- Values * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ by Student's t-test.

5.3.2 Down-stream targets of PERK and ATF6 Pathway are Up-regulated by Tun and SFA: Sal Alleviates ER Stress Response

Grp78/Bip was significantly increased in SFA treated cells (**Figures. 5.3.2.1**). Sal significantly down-regulated the expression of Grp78/Bip in SFA treated cells (**Figures. 5.3.2.1**).



Another protein chaperone, Calnexin, was significantly up-regulated by SFA, which was significantly down-regulated by Sal (**Figure. 5.3.2.4**). Sal alone also significantly suppressed the Calnexin expression compared with control.

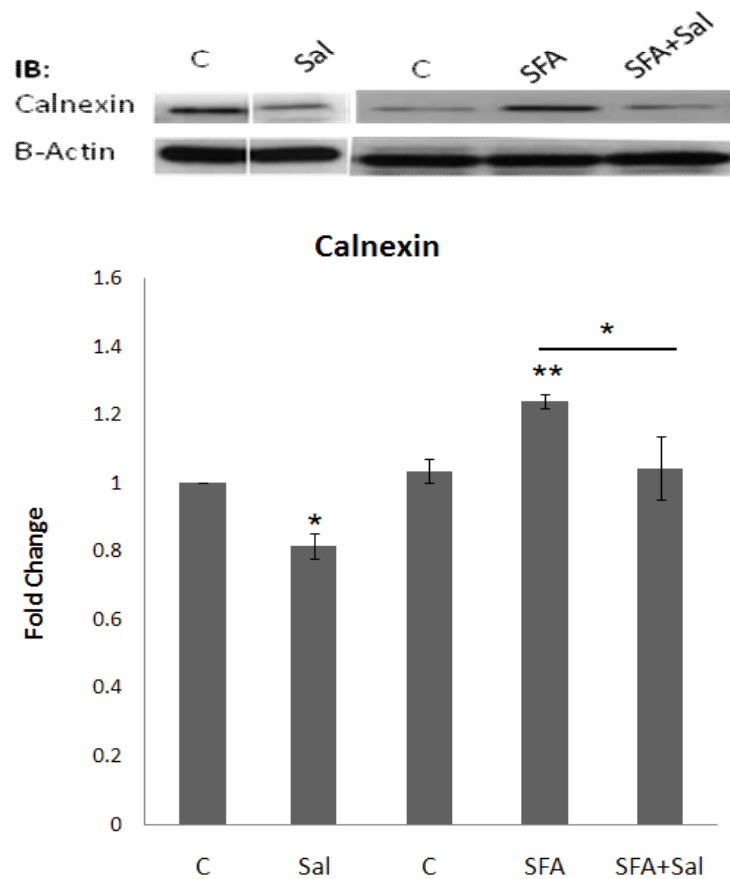


Figure 5.3.2.2 Protein expression levels of Calnexin in SFA treated adipocytes, either alone or in combination with Sal, as measured by Western blot. In the figure, values are presented as the mean \pm SEM expressed as a relative fold difference to control, which was given an arbitrary value of 1. P-Values * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ by Student's t-test.

The mRNA expression of protein chaperones, PDI and Ero1- α , were significantly increased in Tun and SFA treated adipocytes (**Figures. 5.3.2.3 & 5.3.2.4**).

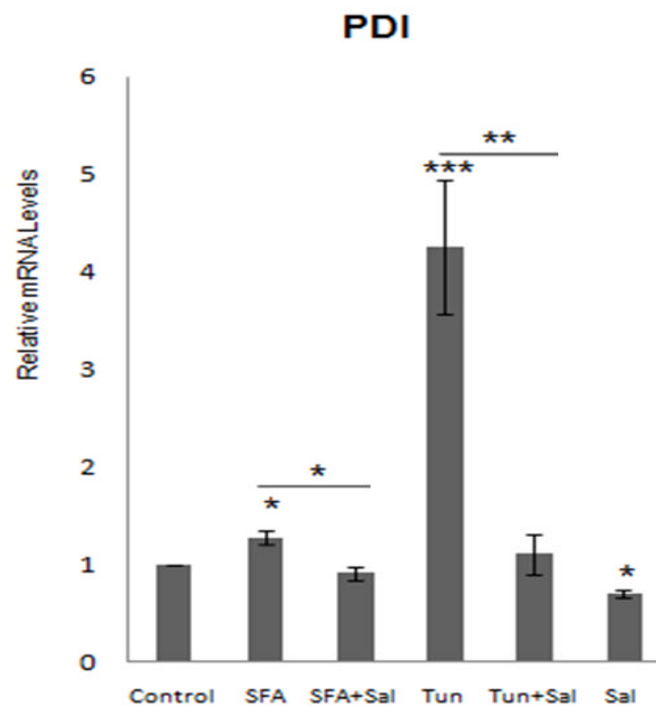


Figure 5.3.2.3 PDI mRNA expression in adipocytes treated with Tun and SFA, either alone or in combination with Sal. The mRNA was determined by qRT-PCR (n=6). In the figure, values are presented as the mean \pm SEM expressed as a relative fold difference to control, which was given an arbitrary value of 1. P-Values * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ by Student's t-test

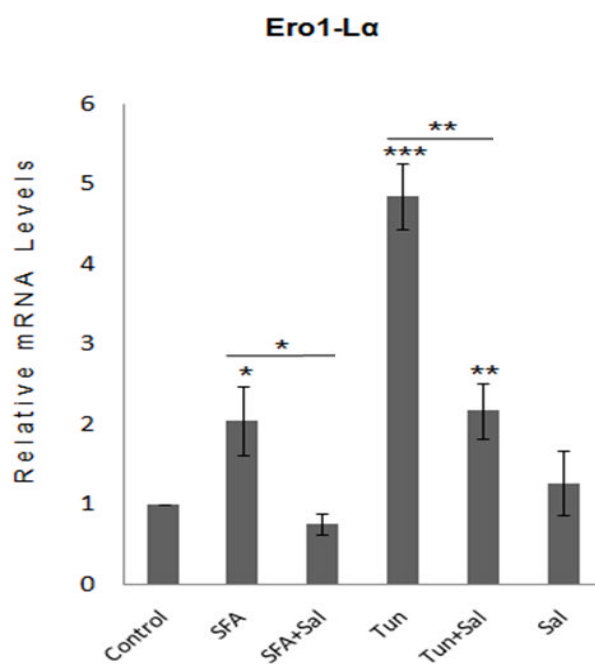


Figure 5.3.2.4 Ero1-L α mRNA expression in adipocytes treated with Tun and SFA, either alone or in combination with Sal. The mRNA was determined by qRT-PCR (n=6). In the figure, values are presented as the mean \pm SEM expressed as a relative fold difference to control, which was given an arbitrary value of 1. P-Values *p<0.05, **p<0.01 and ***p<0.001 by Student's t-test.

In adipose cells the expression of the chaperones was down regulated in response to Salicylate treatment in combination with SFA. To examine this pathway further mRNA expression of the ER stress-induced apoptotic transcription factor, CHOP, in Tun and SFA treated primary adipocytes. All these treatments significantly increased CHOP expression (at least 2-fold) (**Figure. 5.3.2.5**). In adipose cells Sal co-treated with SFA had a more significant impact on down-regulating CHOP expression than Tun and Sal (**Figure. 5.3.2.5**).

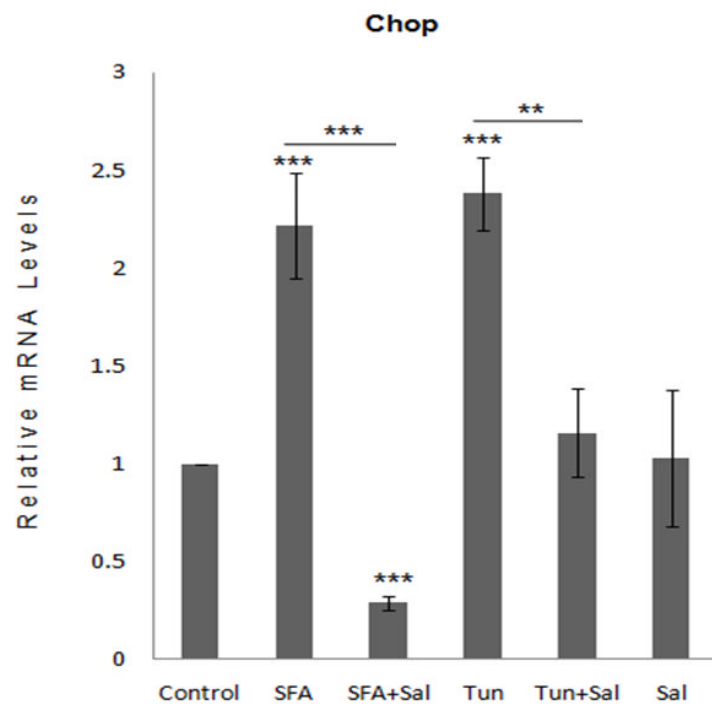


Figure 5.3.2.5 CHOP mRNA expression in adipocytes treated with Tun and SFA, either alone or in combination with Sal. The mRNA was determined by qRT-PCR (n=6). In the figure, values are presented as the mean \pm SEM expressed as a relative fold difference to control, which was given an arbitrary value of 1. P-Values * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ by Student's t-test.

5.3.3 Activation of AktSer473 in Response to High SFA

Akt activation was examined by measuring p-AktSer473 levels in the adipocytes treated with SFA alone and in combination with Sal. SFA showed no effect on the induction of p-AktSer473. There was significant induction by Sal in adipocytes treated with SFA (**Figures: 5.3.3.1**). Sal alone also significantly increased p-AktSer473 levels (**Figures: 5.3.3.1**).

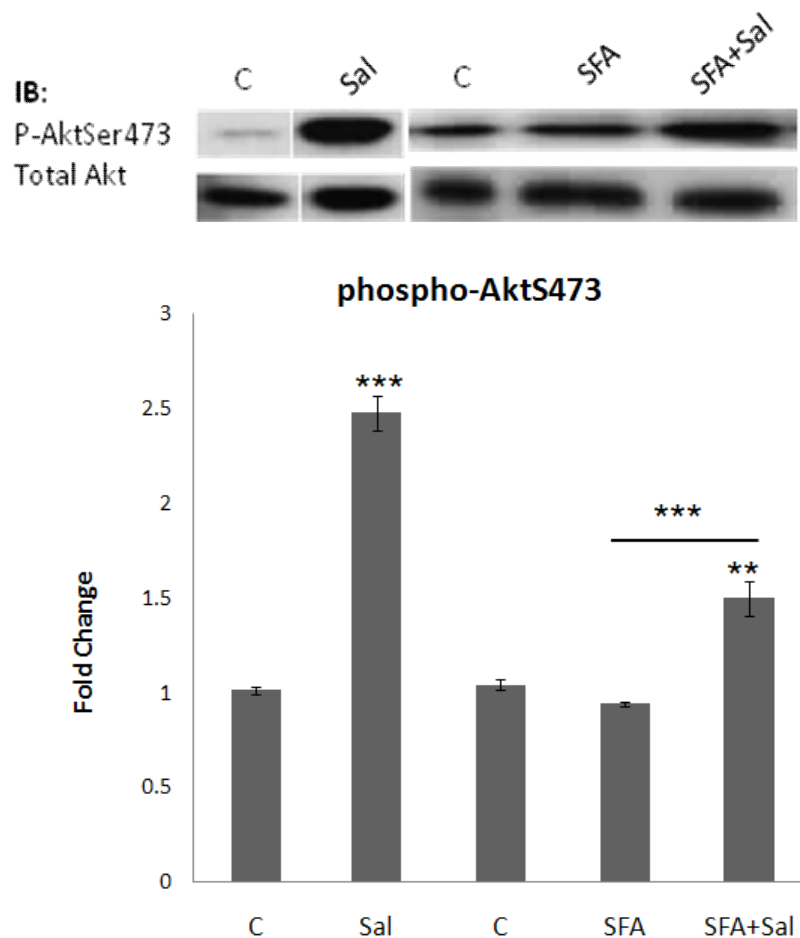


Figure 5.3.3.1 Expression of p-AktSer473. Protein expression levels of p-AktSer473 in SFA treated adipocytes, either alone or in combination with Sal, as measured by Western blot. In the figure, values are presented as the mean \pm SEM expressed as a relative fold difference to control (C), which was given an arbitrary value of 1 (n=3-6). P-Values * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ by Student's t-test.

5.3.4 Secretion of Adipokines in Response to Inducers of Stress

5.3.4.1 IL-6 Secretion in Response to Treatment

Due to the variability in individual human pre-adipocytes compared across treatment regimens, data were expressed as relative fold standardised to control when the Western blot data were examined. From these studies the impact of a known positive inducer of ER stress, Tun, was examined as well as HG (25mM), and high SFA (2mM) alone compared with untreated differentiated AbSc pre-adipocyte cells. Tun, HG and SFA all significantly induced IL-6 secretion (Control: $1(\text{SEM}) \pm 0.0$; Tun: $3.12 \pm 1.03^{**}$; Glucose: $2.88 \pm 0.22^{**}$; SFA: $74.31 \pm 10.33^{***}$; P-Values: $^{**}p < 0.01$ and $^{***}p < 0.001$; **Figure 5.3.4.1**).

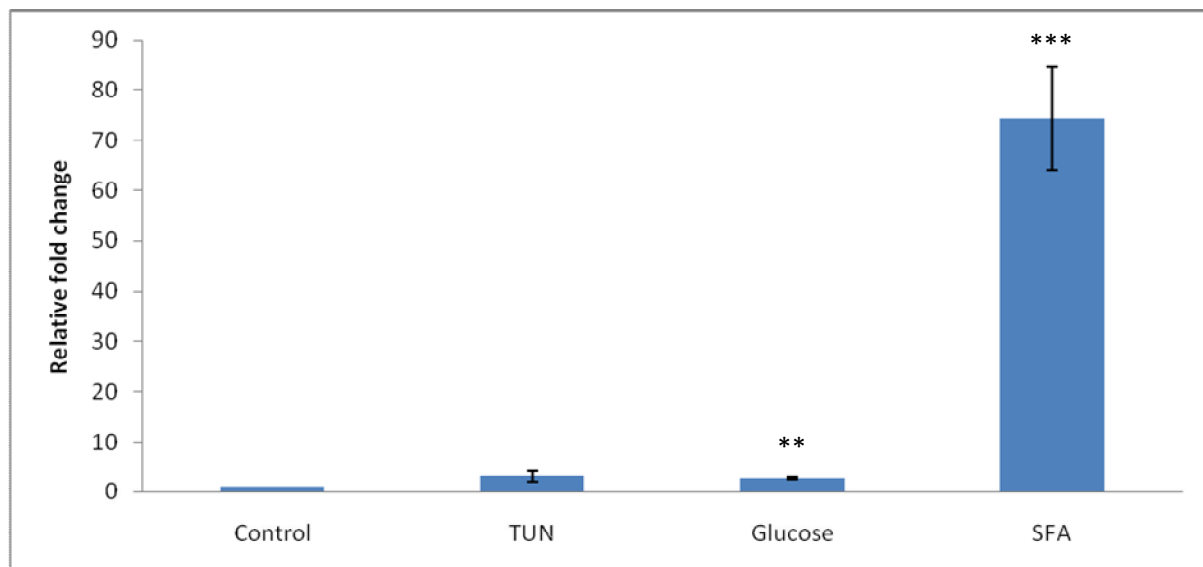


Figure 5.3.4.1 Secretion of interleukin 6 IL-6 in response to treatment. Secretion of IL-6 in Tun (750ng/ml), glucose (25mM) or SFA (2mM) treated differentiated AbSc adipocytes as measured by ELISA. In the graph, values are presented as the mean \pm SEM (n=6). P-Values: $^{**}p < 0.01$ and $^{***}p < 0.001$ by Student's t-test.

5.3.4.2 Leptin Secretion in Response to Treatment

From these studies the impact of leptin secretion was examined following treatment with Tun, HG (25mM) or high SFA (2mM) alone compared with untreated differentiated AbSc pre-adipocyte cells used as a control set and given an arbitrary value of 1. SFA significantly reduced the leptin secretion (Control: $1(\text{SEM}) \pm 0.0$; Tun: 1.50 ± 0.35 ; Glucose: 1.36 ± 0.56 ; SFA: $0.46 \pm 0.23^{**}$; P-Values: $^{**}p < 0.01$; **Figure 5.3.4.2**).

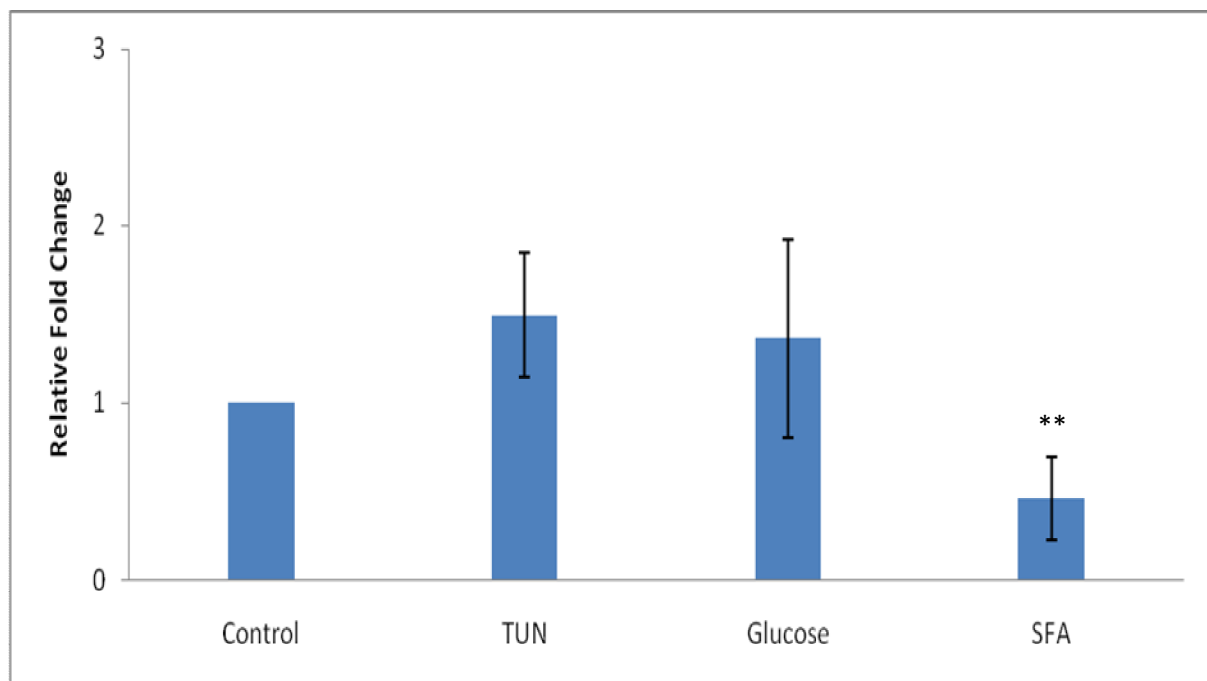


Figure 5.3.4.2 Secretion of leptin in response to treatment. Secretion of leptin in Tun (750ng/ml), glucose (25mM) or SFA (2mM) treated differentiated AbSc adipocytes, as measured by ELISA. In the graph, values are presented as the mean \pm SEM (n=6). P-Values: $^{**}p < 0.01$ by Student's t-test.

5.3.4.3 IL-6 Secretion in Response to SFA, Chemical Chaperones: PBA, TUDCA and Salicylate

For these studies the impact of leptin secretion was examined following treatment with Tun, TUDCA and PBA in the presence of SFA (2mM) alone and SFA in combination with Sal. Untreated differentiated AbSc pre-adipocyte cells were used as a control and set an arbitrary value of 1 for comparison. SFA significantly induced IL-6 secretion. The chemical chaperone PBA didn't have any effect while TUDCA and Sal significantly reduced the IL-6 secretion induced by SFA (Control: $1(\text{SEM}) \pm 0.0$; SFA: $74.31 \pm 10.33^{***}$; SFA/PBA: $76.05 \pm 17.43^{***}$; SFA/TUDCA: $42.10 \pm 9.15^{***}$ SFA/Sal: $10.47 \pm 0.54^{***}$; P-Value: $^{***}p < 0.001$;

Figure 5.3.4.3).

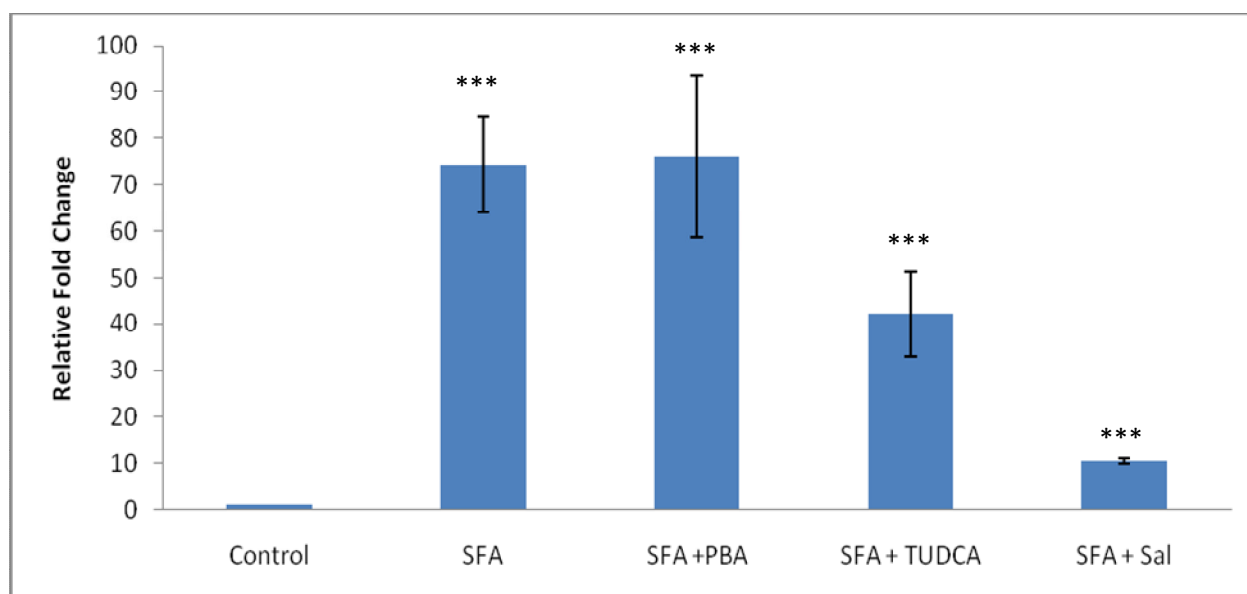


Figure 5.3.4.3 Secretion of IL-6 in response to treatment. Secretion of IL-6 in SFA (2mM) alone or in the presence of 4-Phenyl butyric acid (PBA: 20mM), Taurine-conjugated ursodeoxycholic acid (TUDCA:500 µg/ml), or sodium salicylate (Sal: 20mM) in cultured differentiated AbSc adipocytes as measured by ELISA. In the graph, values are presented as the mean ± SEM (n=6). P-Value: $^{***}p < 0.001$ by Student's t-test.

5.3.4.4 Leptin Secretion in Response to SFA, Chemical Chaperones: PBA, TUDCA and salicylate.

For these studies the impact of leptin secretion was examined following treatment with Tun, TUDCA and PBA in the presence of SFA (2mM) alone and SFA in combination with Sal. Untreated differentiated Abd Sc pre-adipocyte cells were used as a control and set an arbitrary value of one for comparison. SFA significantly reduced the leptin secretion which was further reduced by PBA and Sal while TUDCA had no effect (Control: $1(\text{SEM}) \pm 0.0$; SFA: $0.45 \pm 0.13^{**}$; SFA/PBA: $0.13 \pm 0.10^{***}$; SFA/TUDCA: $0.45 \pm 0.01^{***}$ SFA/Sal: $0.16 \pm 0.12^{***}$; P-Value: $^{***}p < 0.001$; **Figure 5.3.4.4**).

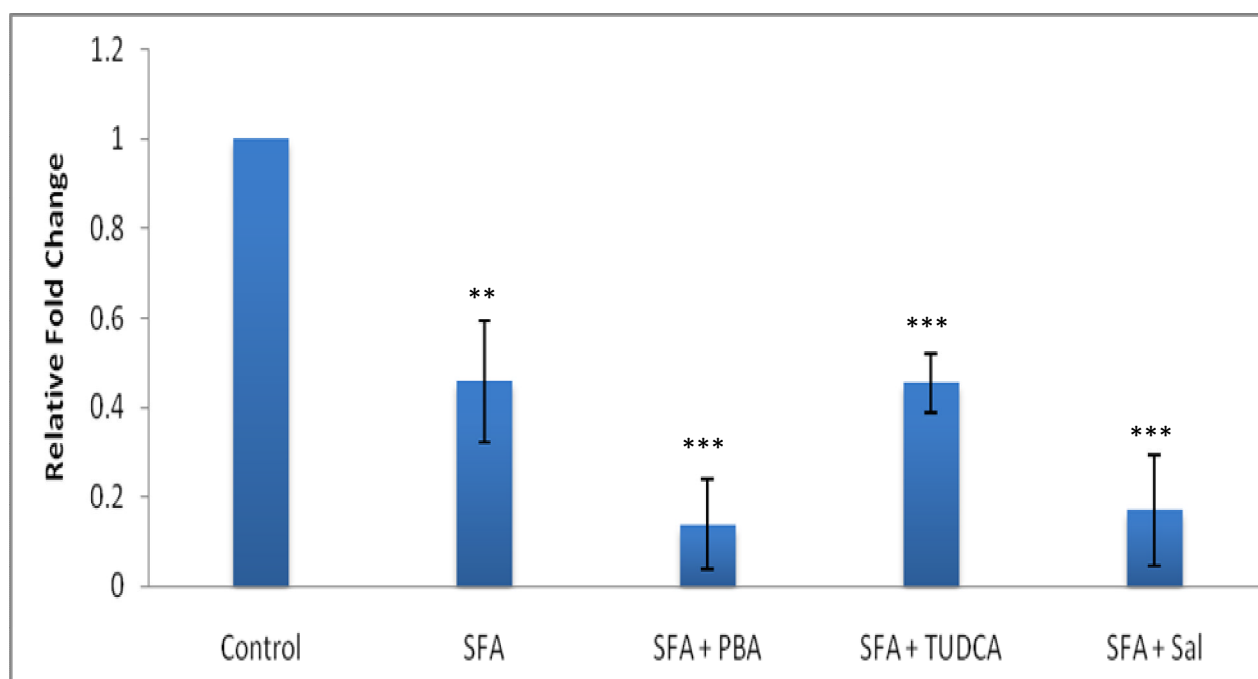


Figure 5.3.4.4 Secretion of leptin in response to treatment. Secretion of Leptin in SFA (2mM) alone or in the presence of 4-Phenyl butyric acid (PBA: 20mM), Taurine-conjugated ursodeoxycholic acid (TUDCA:500 $\mu\text{g/ml}$), or sodium salicylate (Sal: 20mM) in cultured differentiated AbSc adipocytes as measured by ELISA. In the graph, values are presented as the mean \pm SEM (n=6). P-Value: $^{***}p < 0.001$ by Student's t-test.

5.4 DISCUSSION

The results of the present chapter demonstrate that firstly, SFA can induce ER stress related mechanisms. Further that activation of SFA mediated stress can be alleviated by salicylates and therefore could contribute to increased insulin sensitivity in human differentiated adipocytes. Free fatty acids (FFA or SFA) have been shown to be elevated in blood during obesity and T2DM and an ability to alleviate this stress within AT provides proof of concept for the importance of ER stress mechanisms and the significance to reduce them (Boden, 2005; Reinehr *et al.*, 2004).

The differentiated adipocytes were treated with SFA with and without PBA, Tun and TUDCA, as well as SFA with and without Sal. The current data demonstrated that SFA activates the PERK pathway. This activation was measured by looking at the expression levels of p-eIF2 α , a down-stream target of PERK, which was significantly increased with Tun and SFA treated adipocytes and this was downregulated by Sal. PERK is activated in response to accumulation of misfolded proteins in the ER, reducing the rate of protein synthesis through eIF2 α phosphorylation at ser51 to assure proper protein folding (Harding *et al.*, 2000a; Harding *et al.*, 2000b). This also induces the transcription of protein chaperones. P-eIF2 α activation was totally eliminated when SFA treatment was given in combination with sal in a similar fashion to the effect noted with HG.

These studies also addressed the protein expression of chaperones, Calnexin, PDI, Ero1-L α and CHOP, showing them to be induced by Tun and/or SFA, with the induction being significantly down-regulated in the presence of Sal. It has been shown that to up-regulate CHOP transcription the PERK-eIF2 α -ATF4 branch of the UPR is essential (Szegezdi *et al.*, 2006).

Namba and co-authors (Namba *et al.*, 2007) have shown that all the ER stressors they examined, such as thapsigargin and Tun up-regulate ATF6 mRNA expression with the cells over-expressing ATF6 mRNA showing an enhanced ER stress response. Within these current studies, adipocytes treated with Tun induced ATF6 mRNA expression. In addition, treatment with sal significantly down-regulated the ATF6 mRNA expression induced by Tun or SFA, whilst there was no marked reduction in ATF6 mRNA expression with SFA and Sal treatment. The reason for the lack of response may relate to the time points investigated, whilst SFA may have a potent effect early on regarding mRNA regulation, this may have been lost post 24 hrs, further data discussed later examining AKT may also support this notion.

An interesting observation was noted with Grp78/Bip expression. The Grp78/Bip expression was significantly increased by Tun and SFA which was in turn down-regulated by Sal. Despite considerable activation of the UPR, HG did not induce Grp78/Bip expression in contrast to SFA. This is of note as previous work has suggested that PERK is found in a complex with Grp78/Bip in cells without ER stress conditions or in an inactive state (Bertolotti *et al.*, 2000; Zhang *et al.*, 2009). However under SFA conditions, both Grp78/Bip expression and eIF2 α are upregulated in the presence of SFA and its down-stream target CHOP is also activated by SFA. It therefore seems likely, as noted for hyperglycaemic conditions, that another mechanism exists for PERK regulation in SFA conditions.

The present study also investigated whether these UPR inducing factors would have an effect on p-AktSer473 levels. However under these present conditions it seemed p-AktSer473 was unaffected by SFA treatment, which also mirrored the lack of ATF6 mRNA expression. These two observations may be a result of the quick response to SFA inducing an immediate response that is not observed 24 hrs post treatment. Studies by Hu and co-authors

have also observed transient activation of Akt during ER stress, induced by thapsigargin and Tun in MCF-7 cells (Hu *et al.*, 2004).

Finally the down-stream effects on adipokine secretion were analysed to examine whether, in addition to mediating ER stress, mechanisms considered relevant to ER stress, such as NFκB and JNK pathways could be affected by SFA, glucose and mediators known to induce ER stress. Current literature indicates a relationship between ER stress and the NFκB and JNK pathways but the intracellular mechanistic cross-talk between them is unknown. However the current data does highlight that SFA, HG and Tun mediate release of IL-6, with SFA showing a substantial increase in IL-6 compared to the other treatments. In contrast, analysis of leptin secretion indicated that SFA treatment led to a reduction in leptin release, whilst glucose and Tun appeared to have no apparent effect on the adipokine release. These findings appear consistent with previous work examining release of IL-6 and leptin, amongst other cytokines, although the explanation for this was not conjectured (Ryan *et al.*, 2010; Wang *et al.*, 2005). However the differences in adipokine release may highlight the subtle differences in the pro-inflammatory nature of the adipokines. IL-6 is often examined as the first acute pro-inflammatory cytokine in human AT, as this cytokine appears dramatically affected by cellular insults. In contrast, leptin may act as a chronic inflammatory cytokine when such an insult persists - in this case the 24 hr treatment may not have been long enough to see a pronounced response. Further examination of IL-6 response with SFA alone, and in combination with ER stress inducers, noted that all treatment regimens increased IL-6 release significantly and, perhaps, indirectly indicating that known stressors of ER stress as well as SFA can impact on regulation of IL-6. The chemical chaperones PBA and TUDCA are known to reduce ER stress in various cell systems and therefore their effect was examined for IL-6 and leptin release in combination with SFA treatments. The PBA seems to have no effect on IL-6 secretion while TUDCA significantly downregulated the SFA induced IL-6

secretion. In the case of leptin release, neither SFA nor any ER stressor caused up-regulation of leptin - in fact, there appeared to be a specific reduction in leptin secretion. The chemical chaperones had opposite effect to what was seen for IL-6 secretion. The reason for this in the literature is unclear but, again, may indicate leptin's role as more than a pro-inflammatory mediator as well as a potential chronic role in inflammation not observed in the first 24 hrs post insult. In the case of leptin it was PBA which further downregulated the leptin secretion compared to TUDCA. They may be affecting the ER stress pathways by different mechanisms and need to be investigated further. It would be of interest to explore this apparent disparity further, but this was considered beyond the scope of these current studies.

In summary, SFA had a clear impact on ER stress, although it highlighted some subtle differences in the intracellular pathways between the Tun, high glucose and SFA. This chapter may suggest SFA has perhaps a more acute effect on p-AktSer473 and ATF6 mRNA expression which measurement at 24 hr was unable to show. Down-stream signals highlighted a dramatic effect of SFA treatment on IL-6 secretion from adipose cells which suggested that SFA had a considerable impact on pro-inflammatory status. These studies also observed the clear impact of sal, which significantly alleviated ER stress induced by Tun and SFA. Taken together, it appears that SFA may have a more pronounced immediate impact on ER stress than glucose, although with chronic elevation of both, as noted in conditions of diabetes, this may not ultimately lead to much differentiation between disease progression in the long-term.

Chapter 6

The Effect of Lipopolysaccharide Mediating ER Stress in Human Differentiated Abdominal Subcutaneous Preadipocytes

6.1 Introduction

Whilst the role of SFA in increasing inflammation and CVD risk seems implicit, intertwined with this is the potential role of metabolic endotoxaemia (Al-Attas *et al.*, 2009; Baker *et al.*, 2009; Brun *et al.*, 2007; Creely *et al.*, 2007; Ghoshal *et al.*, 2009; Hansson, 2005; Kris-Etherton *et al.*, 2001; Miller *et al.*, 2009; Shoelson and Goldfine, 2009; Winer *et al.*, 2009). In essence this is when gut derived bacteria releases a toxin, known as lipopolysaccharide (LPS) or endotoxin, that has a strong affinity for chylomicrons (lipoproteins that transport dietary long chain SFA through the gut wall), cross the GI mucosa to increase inflammation exacerbated postprandially (Ghoshal *et al.*, 2009). Recent data have highlighted that absorbed gut bacteria, LPS, is raised in patients with T2DM and CVD, which can also affect blood pressure (Al-Attas *et al.*, 2009; Baker *et al.*, 2009; Creely *et al.*, 2007; Fogelstrand *et al.*, 2004; Wei *et al.*, 2007). Additionally, recent human studies have explored the effect of a high fat, high carbohydrate meal on circulating LPS levels in healthy individuals and shown substantially increased circulating LPS, in conjunction with markers of inflammation - as noted from mononuclear blood cells (Barcia and Harris, 2005; Ghanim *et al.*, 2009). Murine studies have also identified an association between LPS and insulin resistance, through infusion of endotoxin, with the same effect also noted by a high fat diet (Cani *et al.*, 2007); with insulin resistance and weight gain both impacting on gut permeability (Brun *et al.*, 2007; Cani *et al.*, 2007; Miller *et al.*, 2009). In addition, previous work has shown that LPS has an immediate impact on human AT, specifically on the innate immune pathway, acting via key receptors known as the toll like receptors (TLRs) which recognise antigens, such as LPS, to initiate an acute phase response to infection (Kaisho and Akira, 2002). Stimulation of the TLRs leads to intracellular activation of NF κ B, a key transcription factor in the inflammatory cascade, that regulates the transcription of numerous pro-inflammatory adipokines (including

IL-6, IL-11, PAI-1, ANG II, resistin and TNF- α) in a rapid response mediated directly by AT, leading to increased metabolic risk factors (Kopp *et al.*, 2009; Lin *et al.*, 2000; Muzio *et al.*, 2000; Song *et al.*, 2006). Both inflammation and the resulting pro-inflammatory adipokines may also lead to increased stress of the ER, which leads to changes in conformation of proteins and dysregulation of normal metabolism, that also contributes to increased metabolic risk in conditions of inflammation (Doroudgar *et al.*, 2009; Feuerer *et al.*, 2009; Gregor and Hotamisligil, 2007; Hotamisligil, 2005; Nishimura *et al.*, 2009; Ozcan *et al.*, 2004; Shoelson and Goldfine, 2009; Wellen and Hotamisligil, 2005; Xu *et al.*, 2009). Supporting evidence for the role of SFA and LPS has indirectly been provided by previous studies examining the impact of Orlistat, a lipase inhibitor, used in the treatment of weight reduction, which leads to both the lowering of circulating LPS and inflammation (Dixon *et al.*, 2008). In effect, both LPS and dietary SFA may simultaneously act on the innate immune pathway to lead to a down-stream pro-inflammatory response via NF κ B, within AT. To support this concept, previous studies have shown a direct correlation between TG and LPS in T2D subjects from a cross-sectional study (Al-Attas *et al.*, 2009). Furthermore, previous correlations have been observed between insulin and endotoxin levels in humans, which may also highlight the importance of insulin's potential to affect gut permeability, as indicated by previous rodent studies (Creely *et al.*, 2007; Kris-Etherton *et al.*, 2001; Miller *et al.*, 2009). Taken together, these studies highlight how subtle postprandial changes, induced by a high fat Western diet, could influence LPS levels. Further, due to the long-term low level nature of these inflammatory processes, abdominal weight gain and, as such, AT could directly contribute to the continued pathogenesis of T2D and CVD through LPS induced ER stress (Hansson, 2005; Shoelson *et al.*, 2006). Therefore the aim of this chapter was to establish whether *in vitro* low levels of LPS can induce ER stress pathways, and can this be alleviated by Salicylates as shown for SFA and glucose.

6.2 Research Design and Methods

6.2.1 Subjects. Human AbSc AT was collected from patients undergoing elective or liposuction surgery, with informed consent obtained in accordance with LREC guidelines and with ethics committee approval. All tissue samples were flash frozen and/or utilised for isolation of stromal fraction, subsequently used for culturing primary human adipocytes as detailed (McTernan *et al.*, 2002).

6.2.2 Cell Culture

AbSc AT was digested with collagenase (Worthington Biochemical, Reading, USA), as previously described, to isolate mature adipocytes and pre-adipocytes (Harte *et al.*, 2003). Firstly, stromal fractions of Human AbSc AT (BMI $25.04 \pm 0.6 \text{ kg/m}^2$; $n = 3-6$) were cultured in tissue culture flasks until confluent and then trypsinised to obtain cells to carry out the study. The preadipocytes from the same passage were grown in 6-well plates to confluence in DMEM/Ham's F-12 phenol-free medium (Invitrogen, Paisley, UK) containing 10% FCS, penicillin (100 U/ml), streptomycin (100 $\mu\text{g/ml}$) and transferrin (5 $\mu\text{g/ml}$). At confluence, preadipocytes were differentiated in preadipocyte differentiation media (Promocell, Germany) containing biotin (8 $\mu\text{g/ml}$), insulin (500 ng/ml), Dexamethasone (400 ng/ml), IBMX (44 $\mu\text{g/ml}$), L-Thyroxine (9 ng/ml) and Ciglitazone (3 $\mu\text{g/ml}$) for 72 hrs. After this period, the differentiating cells were grown in nutrition media containing DMEM/Ham's F-12, 3% FCS, d-biotin (8 $\mu\text{g/ml}$), insulin (500 ng/ml) and Dexamethasone (400 ng/ml) until fully differentiated (14-18 days). The viability of adipocytes was assessed using the trypan blue dye exclusion method (Sigma-Aldrich Corp., Poole, UK) (Matthews *et al.*, 1985).

6.2.3 Treatments

Fully differentiated adipocytes (day 15) were grown in normal DMEM/Ham's F-12 phenol-free medium containing only 2% serum (detoxification media) for 24 hours to remove effects of growth factors and other components in nutrition media. The treatments were then placed in the fresh detoxification media for 24 hrs. The cells were treated with LPS (100ng/ml), tunicamycin (Tun: 750ng/ml); and sodium salicylate (Sal) (20mM) for 24 hrs. All the data shown in this study is based on a 24 hr treatment.

6.2.4 Quantitative Measurement of Secreted Adipokines Concentration From Treated Differentiated Predipocytes

Conditioned media from untreated, cultured, differentiated adipocytes (control) and from treated differentiated adipocytes, were assayed for leptin and IL-6 using human ELISA kits (leptin, IL-6; Hertfordshire, Millipore, UK). Conditioned media were analysed using a solid phase enzyme-linked immunosorbant assay, the leptin ELISA had an assay limit of 0.5 ng/ml, intra assay coefficient of variance (CV) of 2.6-4.6% and inter-assay variability was 1.4-4.9%; IL-6 ELISA had an assay limit of 0.3 pg/ml, intra assay coefficient of variance (CV) of 4.5-6.9% and inter-assay variability was 6.2-8.0%. Methodology for the full protocol of a standard ELISA is given in the materials and methods.

6.2.5 Immunoblotting

As outlined previously, cells were washed in PBS and harvested in 250 µl of lysis buffer (20 mM Tris-HCl, pH 7.5; 137 mM NaCl; 1mM EGTA, pH 8; 1% Triton X-100; 10% glycerol;

1.5mM MgCl₂) containing protease and phosphatase inhibitors (10 mM NaF; 1 mM PMSF; 1 mM sodium metavanadate; 5 µg/ml aprotinin; 10 µg/ml leupeptin) and stored at -80°C until required (Harte *et al.*, 2003). Proteins (10-20 µg) from cell lysates were resolved by SDS-PAGE and transferred to an Immobilon-P membrane (Millipore, Hertfordshire, UK) by electroblotting. The membranes were blocked with 0.2% I-BlockTM (Applied Biosystems, Warrington, UK); 0.1% Tween20 in PBS and probed with primary and secondary antibodies. Primary antibodies were: phospho and total Akt, phospho and total eIF2 α , Bip/GRP78, Calnexin, Protein disulfide isomerase (PDI), Ero1-L α , phospho-PERK (p-PERK), IRE1 α and β -actin (Cell Signalling Technologies, Massachusettes, USA). Antigen-antibody complexes were visualised using ECL reagents (Amersham, UK). Scanned autoradiographs were semi-quantified using 2D densitometry software (GeneTools, Syngene, Cambridge, UK). The bands were first normalised as a function of the loading control (protein of interest/ β -actin) or total expression of the proteins (for phosphor proteins), then converted to fold change compared with controls.

6.2.6 Extraction of RNA and Quantitative RT-PCR

To characterise gene expression, RNA was extracted from differentiated adipocytes, according to manufacturers' instructions (RNeasy Lipid Tissue Mini Kit, Qiagen, Crawley, UK). Following DNase treatment and reverse transcription, mRNA expression levels were determined using an ABI 7500 Real-time PCR Sequence Detection system. Pre-optimised quantitative primer and probe sequences for genes were utilised (Applera, Cheshire, UK). All reactions were multiplexed with the housekeeping gene 18S, provided as a pre-optimised control probe (Applera, Cheshire, UK), enabling data to be expressed in relation to an internal reference to allow for differences in relative threshold efficiency. Data were obtained

as cycle threshold (Ct) values (the cycle number at which logarithmic PCR plots cross a calculated threshold line) and used to determine ΔCt values ($\Delta\text{Ct} = \text{Ct of 18S housekeeping gene} - \text{Ct of gene of interest}$). Measurements were carried out on at least three occasions for each sample. To exclude potential bias due to averaging, data have been transformed through the Power equation $2^{-\Delta\Delta\text{Ct}}$, all statistics were performed at this stage.

6.2.7 Statistical analysis

Data in the text and figures are presented as mean \pm standard error of the mean (SEM) for at least three independent experiments performed in triplicates to ensure reproducibility. Students *t* test was used to compare values between two groups unless stated otherwise. *P* values <0.05 were considered to represent statistically significant differences.

6.3 RESULTS

6.3.1 PERK and ATF6 Regulation in Response to LPS

The protein, p-eIF2 α , is the down-stream-target of p-PERK and was significantly induced by LPS and Tun – both of which significantly induced the p-eIF2 α expression compared with control; Tun treated cells were used as a comparison with LPS treated cells (**Figure 6.3.1.1**). In contrast, Sal significantly down-regulated the expression of p-eIF2 α in adipocytes treated with LPS and Tun (**Figure 6.3.1.1**). There was no change in the total eIF2 α expression levels.

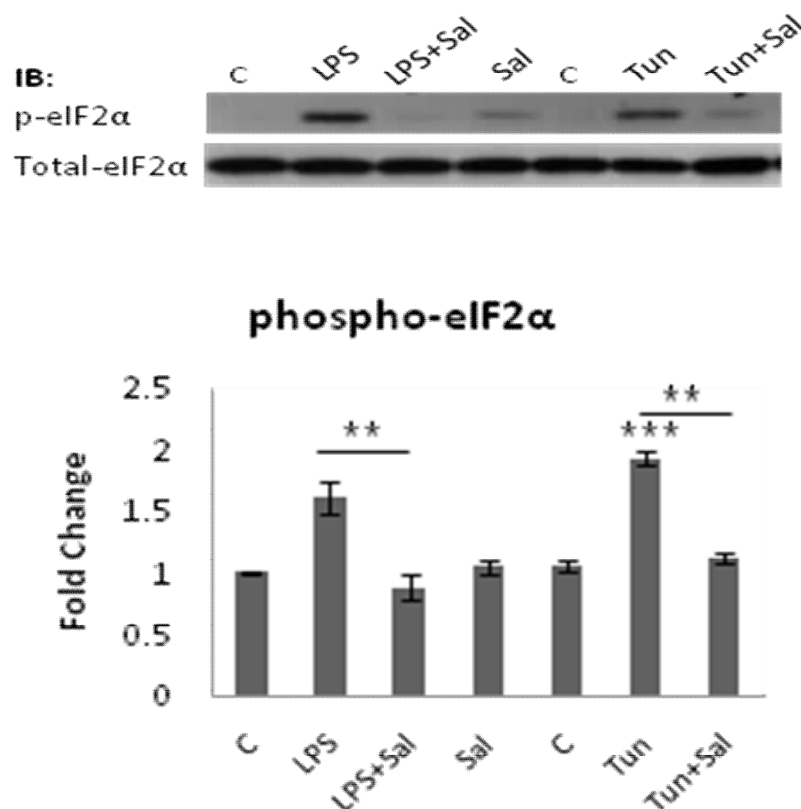


Figure 6.3.1.1 Protein expression of p-eIF2 α and total p-eIF2 α in LPS or Tun treated adipocytes either alone or in combination with Sal (n=3-6). In the figure, values are presented as the mean \pm SEM expressed as a relative fold difference to control, which was given an arbitrary value of 1. P-Values: **p<0.01 and ***p<0.001 by Student's t-test.

The ATF6 mRNA level was significantly increased in the cells treated with LPS and Tun (Figure 6.3.1.2). ATF6 mRNA levels were significantly down-regulated in adipocytes treated with LPS and Tun, in combination with Sal and Sal alone (Figure 6.3.1.2).

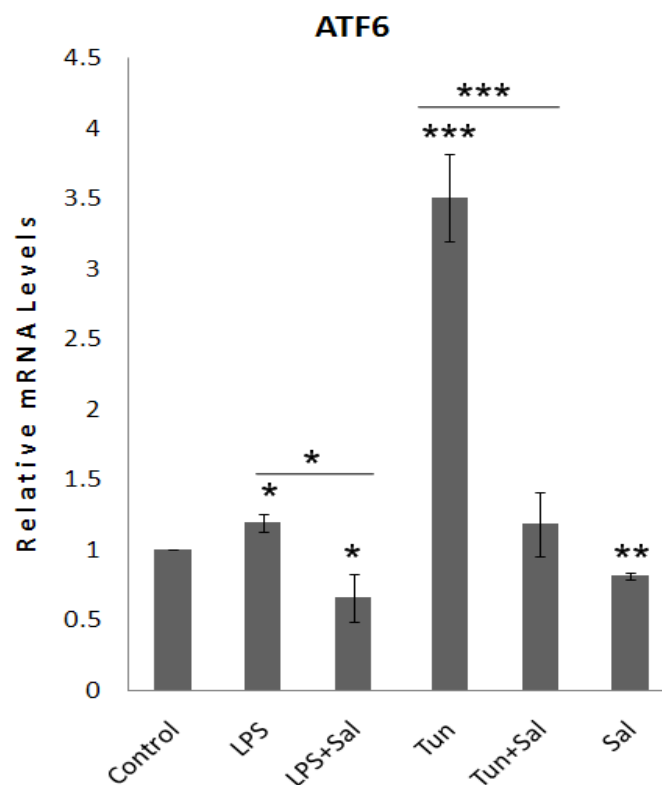


Figure 6.3.1.2 ATF6 mRNA expression in adipocytes treated with LPS or Tun, either alone or in combination with Sal. The mRNA was determined by qRT-PCR (n=6). In the figure, values are presented as the mean \pm SEM expressed as a relative fold difference to control, which was given an arbitrary value of 1. P-Values: * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ by Student's t-test.

6.3.2 Down-stream targets of PERK and ATF6 pathway are up-regulated by Tun and LPS: Sal alleviates ER stress response

Grp78/Bip was significantly increased in Tun treated cells only (**Figure. 6.3.2.1**). There was an increase in LPS treated cells but this was not significant. Sal significantly down-regulated the expression of Grp78/Bip in Tun treated cells (**Figure. 6.3.2.1**).

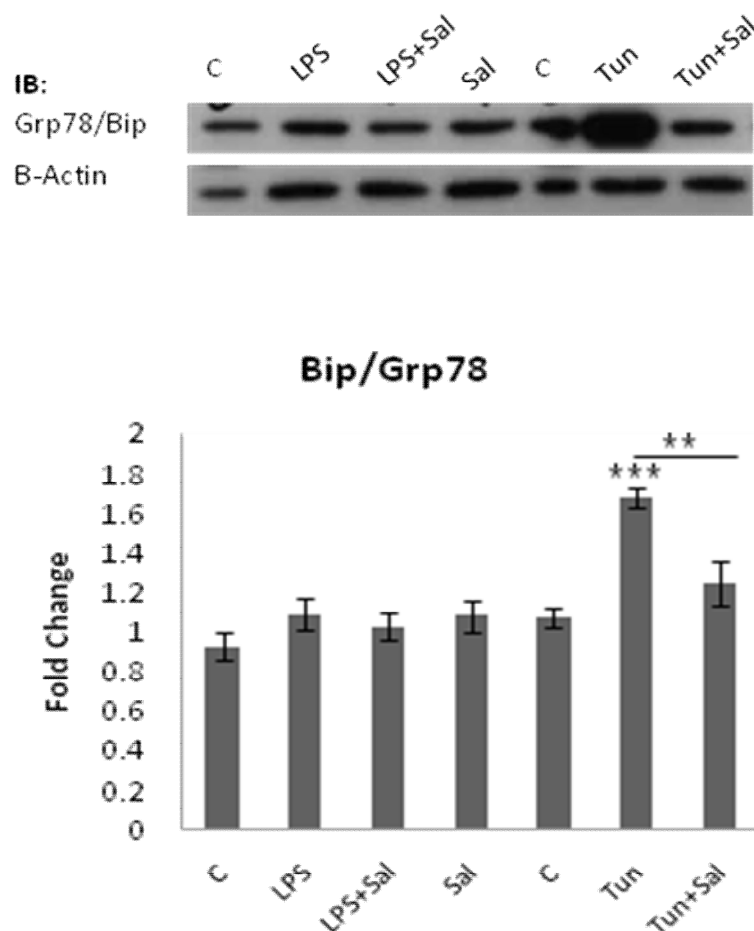


Figure 6.3.2.1 Protein expression levels of Grp78/Bip in LPS or Tun treated adipocytes, either alone or in combination with Sal, as shown by Western blot (n=3-6). In the figure, values are presented as the mean \pm SEM expressed as a relative fold difference to control, which was given an arbitrary value of 1. P-Values: ** $p < 0.01$ and *** $p < 0.001$ by Student's t-test.

Another protein chaperone, Calnexin, was significantly up-regulated by LPS and Tun but this effect was significantly down-regulated by Sal (**Figure. 6.3.2.2**). Sal alone also significantly suppressed Calnexin expression compared with the control.

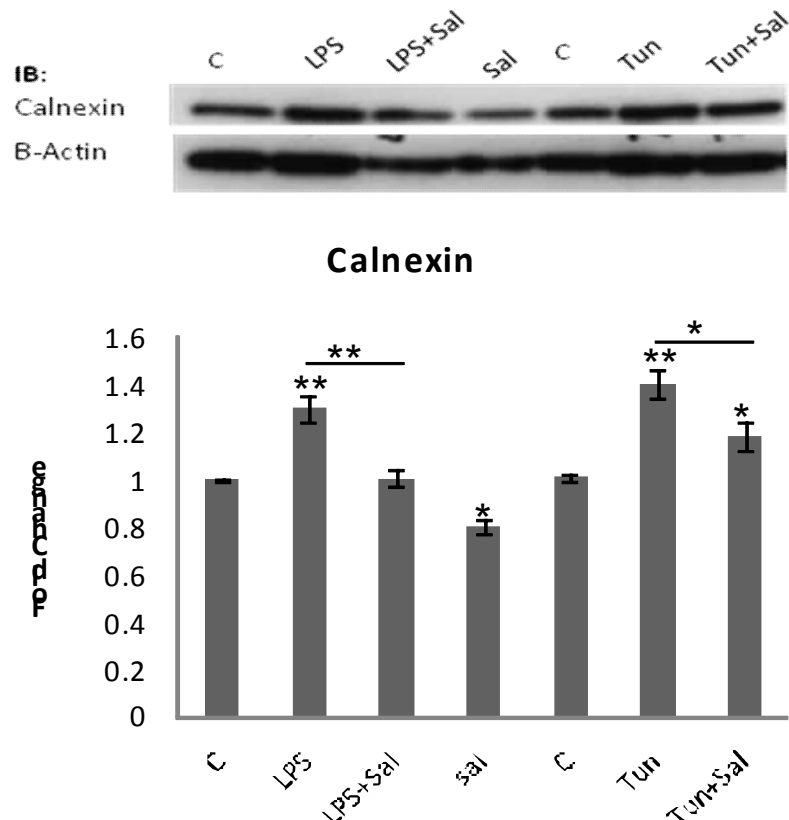
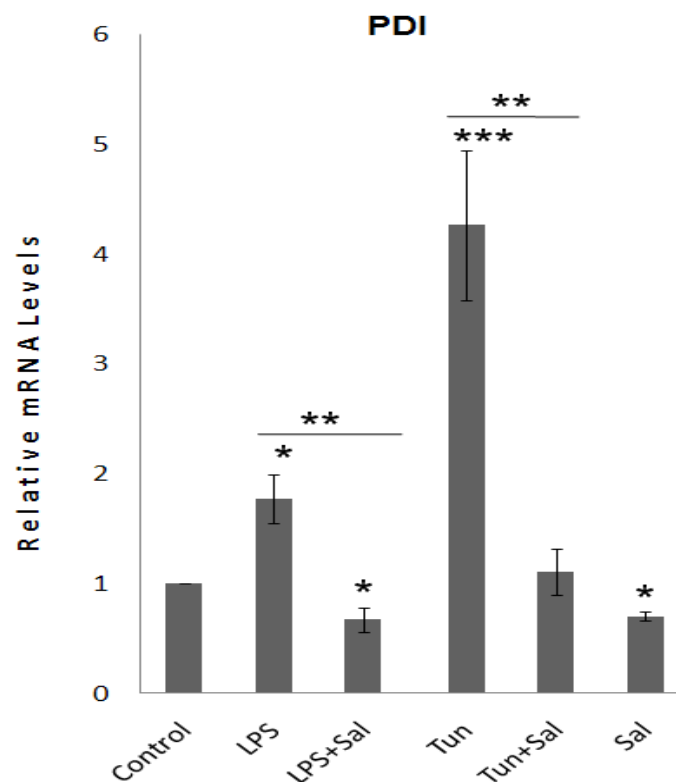


Figure 6.3.2.2 Protein expression levels of Calnexin in LPS or Tun treated adipocytes, either alone or in combination with Sal, as measured by Western blot. In the figure, values are presented as the mean \pm SEM expressed as a relative fold difference to control, which was given an arbitrary value of 1. P-Values: * $p < 0.05$, ** $p < 0.01$ by Student's t-test

The mRNA expression of the protein chaperones PDI and Ero1- α were significantly increased in LPS and Tun treated adipocytes (**Figures: 6.3.2.3 & 6.3.2.4**). Sal, again, significantly down-regulated the expression of these chaperones in the adipocytes treated in combination with Sal, with Sal alone significantly down-regulating PDI mRNA expression levels (**Figure. 6.3.2.3**).



Figures: 6.3.2.3 PDI mRNA expression in adipocytes treated with LPS or Tun, either alone or in combination with Sal. The mRNA was determined by qRT-PCR (n=6). In the figure, values are presented as the mean \pm SEM expressed as a relative fold difference to control, which was given an arbitrary value of 1. P-Values *p<0.05, **p<0.01 and ***p<0.001 by Student's t-test.

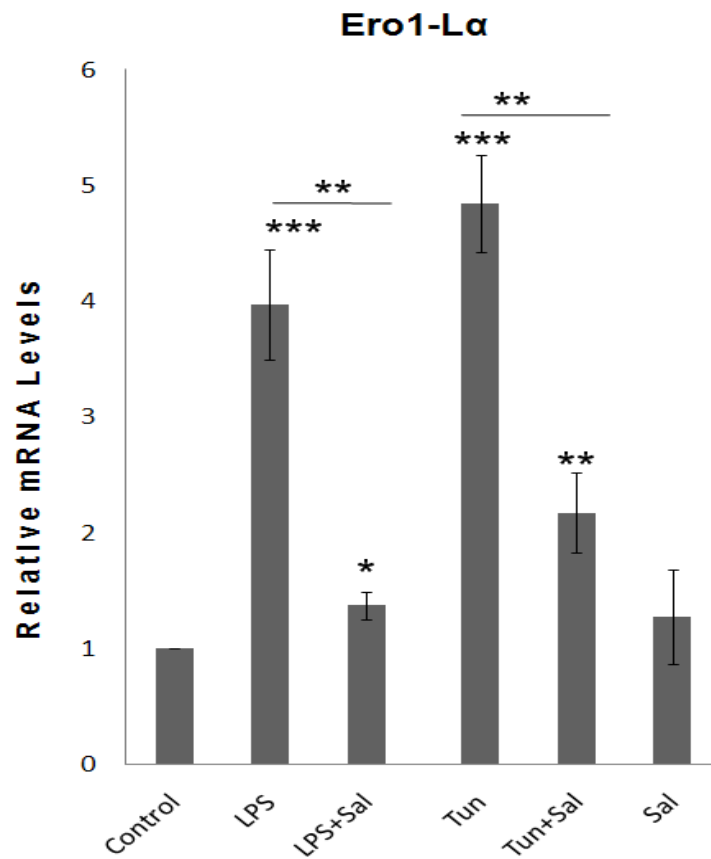


Figure 6.3.2.4 Ero1-L α mRNA expression in adipocytes treated with LPS or Tun, either alone or in combination with Sal. The mRNA was determined by qRT-PCR (n=6). In the figure, values are presented as the mean \pm SEM expressed as a relative fold difference to control, which was given an arbitrary value of 1. P-Values *p<0.05, **p<0.01 and ***p<0.001 by Student's t-test.

Signalling through PERK, ATF6 and IRE1 α can induce pro-apoptotic signals during extreme ER stress, by activating down-stream targets such as CHOP. Therefore, we examined mRNA expression of the ER stress-induced apoptotic transcription factor CHOP in LPS treated primary adipocytes using Tun treated cells as a comparison. Both of these treatments significantly increased CHOP expression (at least 2-fold) (**Figure. 6.3.2.5**). Interestingly, Sal significantly down-regulated CHOP expression induced by LPS and Tun (**Figure.6.3.2.5**).

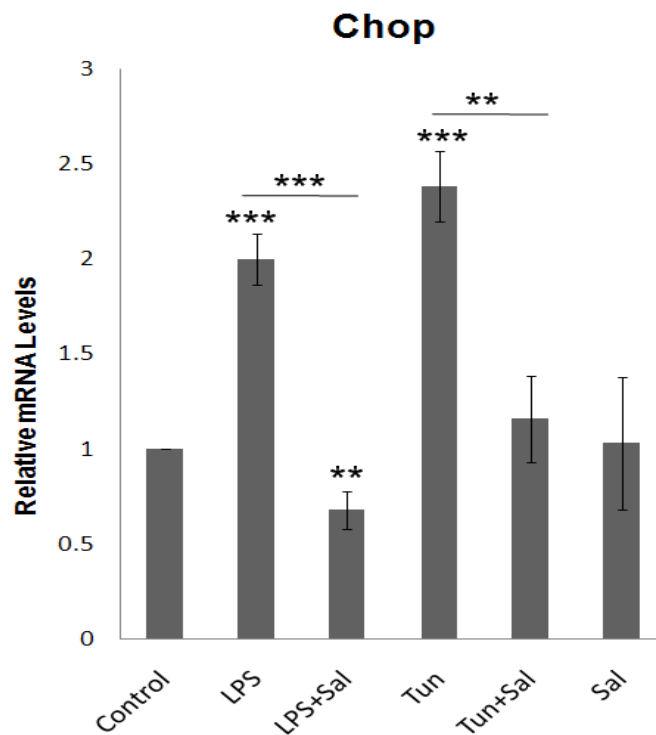


Figure 6.3.2.5 CHOP mRNA expression in adipocytes treated with LPS and Tun, either alone or in combination with Sal. The mRNA was determined by qRT-PCR (n=6). In the figure, values are presented as the mean \pm SEM expressed as a relative fold difference to control, which was given an arbitrary value of 1. P-Values: **p<0.01 and ***p<0.001 by Student's t-test.

6.3.3 Activation of AktSer473 in Response to LPS

Akt activation was examined by measuring p-AktSer473 levels in the adipocytes treated with LPS and Tun alone and in combination with Sal. LPS and Tun treated cells significantly induced p-AktSer473 levels compared to control. In contrast there was a significant induction by Sal in adipocytes treated with LPS and Tun (**Figure. 6.3.3.1**). Whilst Sal alone also significantly increased p-AktSer473 levels

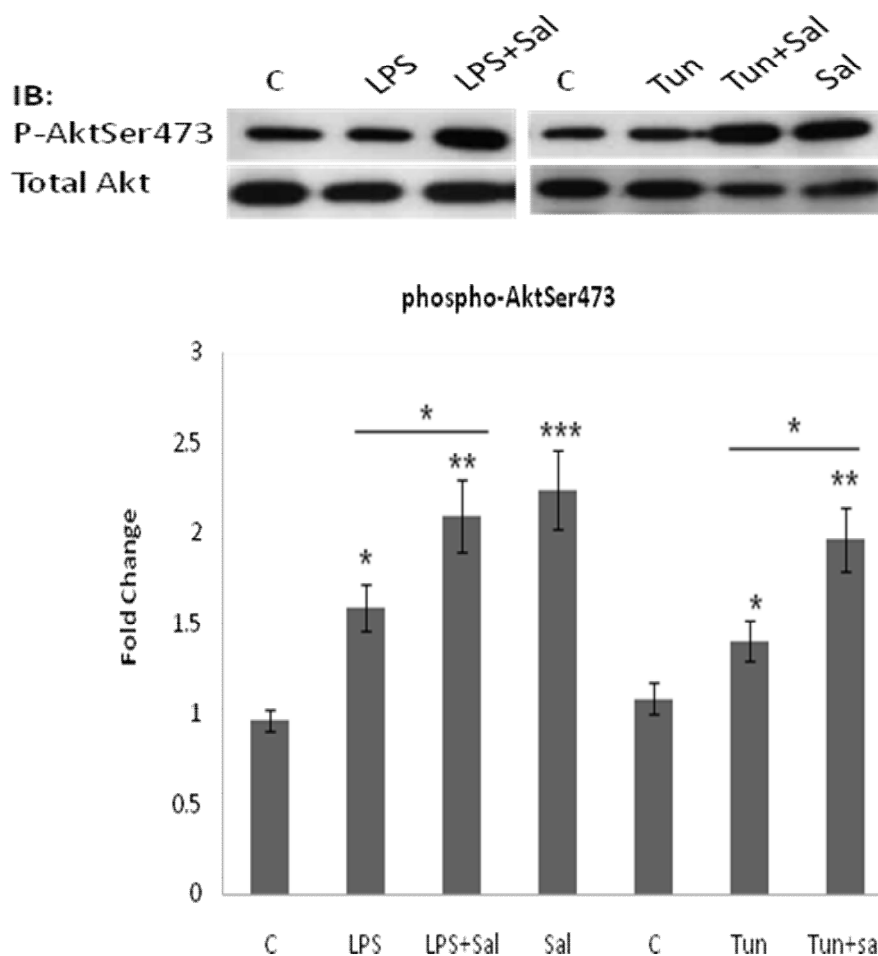


Figure. 6.3.3.1 Protein expression levels of p-AktSer473 in SFA treated adipocytes, either alone or in combination with Sal, as measured by Western blot. In the figure, values are presented as the mean \pm SEM expressed as a relative fold difference to control (C), which was given an arbitrary value of 1 (n=3-6). P-Values * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ by Student's t-test.

6.3.4 IL-6 Secretion in Response to Treatment

Due to the variability in individual human pre-adipocytes compared across treatment regimens, data are expressed as relative fold compared with control, as previously shown with Western blot data analysis. From these LPS studies, a comparison was undertaken with a known positive inducer of ER stress, Tun, as well as HG (25mM) and high SFA (2mM) alone compared with untreated differentiated AbSc pre-adipocyte cells (control), set an arbitrary value of 1 (Control: $1(\text{SEM}) \pm 0.0$; Tun: $3.12 \pm 1.03^{**}$; Glucose: $2.88 \pm 0.22^{**}$; LPS: $41.38 \pm 7.12^{***}$ SFA: $74.31 \pm 10.33^{***}$; P-Values: $^{**}p < 0.01$ and $^{***}p < 0.001$; **Figure 6.3.4.1**).

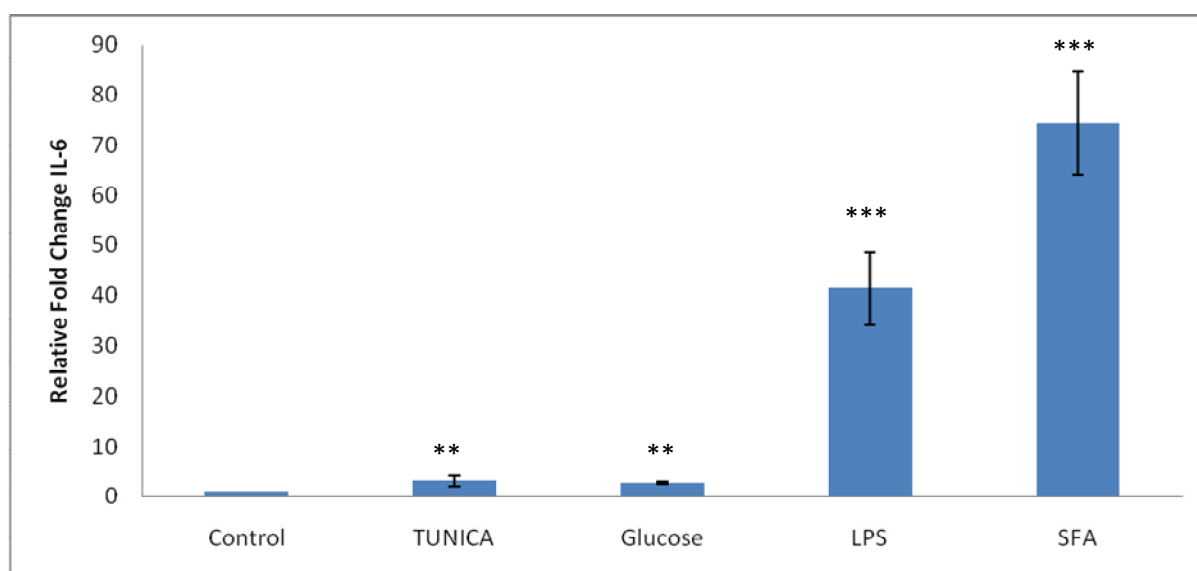


Figure 6.3.4.1 Secretion of IL-6 in Tun (750ng/ml), glucose (25mM), LPS (100ng/ml) or SFA (2mM) treated differentiated AbSc adipocytes, as measured by ELISA. In the graph, values are presented as the mean \pm SEM (n=6). P-Values: $^{**}p < 0.01$, $^{***}p < 0.001$ by Student's t-test.

Further studies investigated the impact of known chemical chaperones shown to reduce ER stress, TUDCA and PBA, in the presence of LPS, as well as alone, in cultured adipose cells. Untreated differentiated AbSc pre-adipocyte cells were used as a control and set an arbitrary value of 1 for comparison (Control: $1(\text{SEM}) \pm 0.0$; LPS: $41.38 \pm 7.12^{**}$; LPS/PBA: $44.54 \pm 10.83^*$; LPS/TUDCA: $13.460 \pm 2.04^{**}$; P-Value: $***p < 0.001$; **Figure 6.3.4.2**).

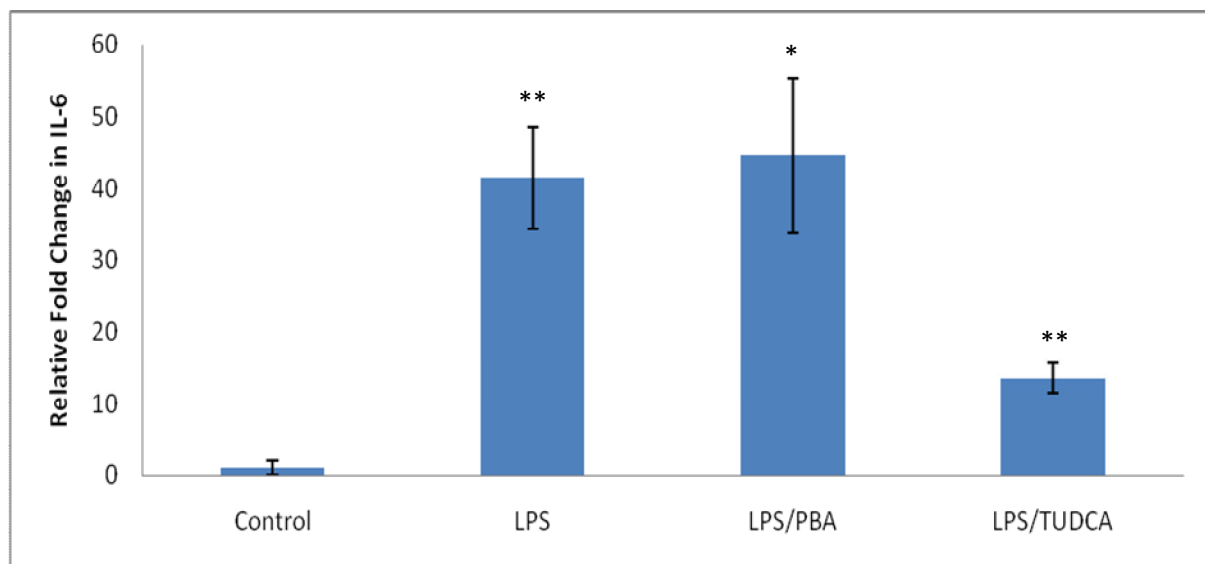


Figure 6.3.4.2 Secretion of IL-6 in LPS (100ng/ml) alone or in the presence of 4-Phenyl butyric acid (PBA: 20mM), Taurine-conjugated ursodeoxycholic acid (TUDCA: 500 $\mu\text{g/ml}$) in cultured differentiated AbSc adipocytes, as measured by ELISA. In the graph, values are presented as the mean \pm SEM (n=6). P-Values: $*p < 0.05$, $**p < 0.01$ by Student's t-test.

6.3.5 Leptin Secretion in Response to Treatment

For these LPS studies a comparison was undertaken with Tun, as well as HG (25mM), and high SFA (2mM) alone compared with untreated differentiated AbSc pre-adipocyte cells, used as a control and set an arbitrary value of 1 for comparison (Control: $1(\text{SEM}) \pm 0.0$; Tun: 1.49 ± 0.35 ; Glucose: 1.36 ± 0.56 ; LPS: 131.039 ± 7.12 ; SFA: $0.45 \pm 0.23^{**}$; P-Values: $^{**}p < 0.01$; **Figure 6.3.5.1**).

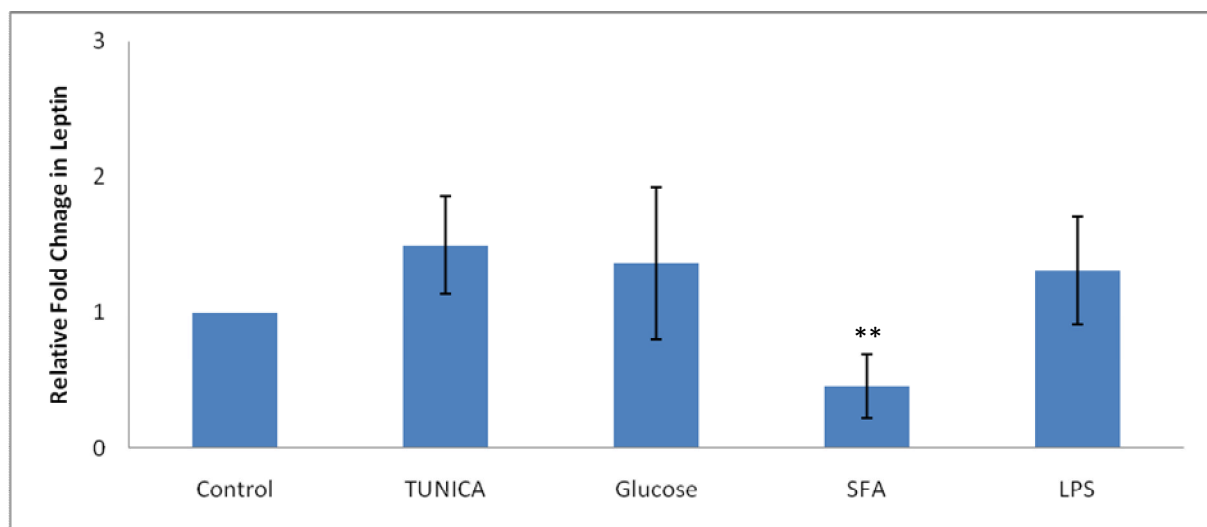


Figure 6.3.5.1 Secretion of leptin in Tun (750ng/ml), glucose (25mM) SFA (2mM) and LPS (100ng/ml) treated differentiated AbSc adipocytes as measured by ELISA. In the graph, values are presented as the mean \pm SEM (n=6). P-Values: $^{**}p < 0.01$ by Student's t-test.

Further studies investigated the impact of TUDCA and PBA, in the presence of LPS, in cultured adipose cells in response to leptin secretion. Untreated differentiated AbSc pre-adipocyte cells were used as a control and set an arbitrary value of 1 for comparison (Control: $1(\text{SEM}) \pm 0.0$; LPS: 1.31 ± 0.19 ; LPS/PBA: $0.41 \pm 0.01^{**}$; LPS/TUDCA: 0.78 ± 0.01 ; P-Value: $***p < 0.001$; **Figure 6.3.5.2**).

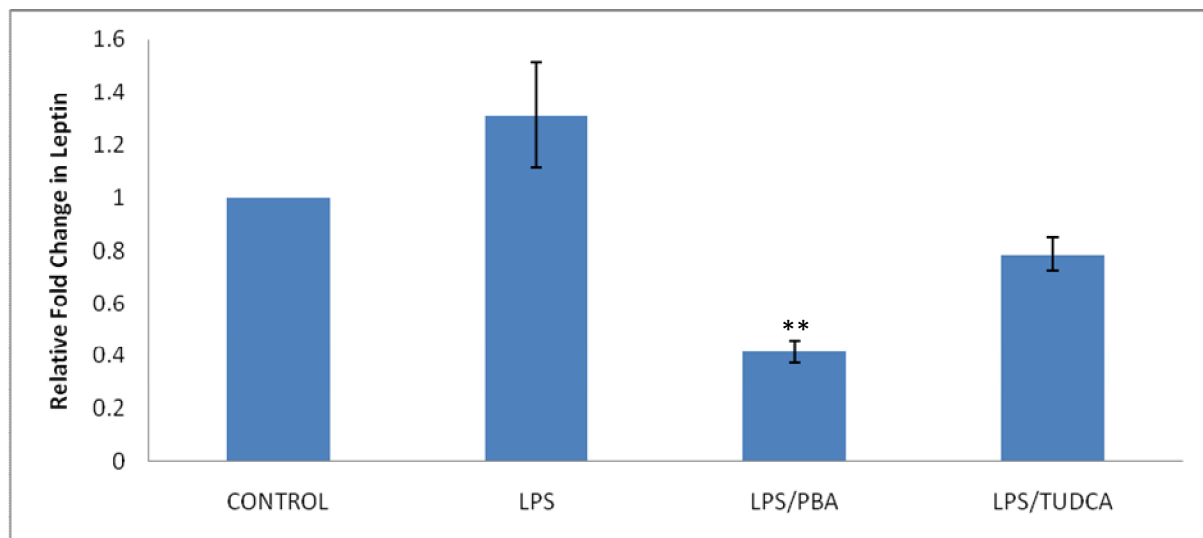


Figure 6.3.5.2 Secretion of leptin in LPS (100ng/ml) alone or in the presence of 4-Phenyl butyric acid (PBA: 20mM), Taurine-conjugated ursodeoxycholic acid (TUDCA: 500 µg/ml) in cultured differentiated AbSc adipocytes, as measured by ELISA. In the graph, values are presented as the mean ± SEM (n=6). P-Value: $^{**}p < 0.01$ by Student's t-test.

6.4 DISCUSSION

This chapter sought to examine the potential role of gut derived endotoxin and its potential impact on ER stress in cultured differentiated pre-adipocytes. Whilst aware that other *in vitro* studies tend to use milligram doses of LPS, these studies examined much lower doses to ascertain the potential effects low dose could have on intracellular metabolism. The results of the present chapter demonstrated that, in a similar manner to SFA, LPS could induce ER stress related mechanisms. Further that activation of LPS mediated stress could be reduced by sal. The role of LPS as a mediator of inflammation and insulin resistance has only been recently studied as previous human studies have highlighted that in states of diabetes and CVD there are raised circulating LPS levels (Al-Attas *et al.*, 2009; Baker *et al.*, 2009; Brun *et al.*, 2007; Creely *et al.*, 2007; Miller *et al.*, 2009).

The differentiated adipocytes were treated with LPS, with and without Sal, and compared with the effects noted for Tun, with and without Sal, for the ER stress pathway. The current data demonstrated that LPS activated the PERK pathway. This activation was measured by looking at the expression levels of p-eIF2 α , a down-stream target of PERK which was significantly increased with LPS treated adipocytes. P-eIF2 α activation was reduced when LPS treatment was given in combination with sal in a similar fashion to the effect noted with SFA or HG.

These studies also addressed the protein expression of the chaperones. Calnexin, PDI, Ero1-L α and CHOP expression were induced by Tun and or/LPS, with the induction being significantly down-regulated in the presence of Sal. It has been shown that to up-regulate CHOP transcription the PERK-eIF2 α -ATF4 branch of the UPR is essential (Szegezdi *et al.*, 2006). Signalling through PERK, ATF6 and IRE1 α can also induce pro-apoptotic signals during extreme ER stress by activating down-stream targets such as CHOP.

Modification of ATF6 protein is also important for the ER stress response. As discussed in earlier chapters, ER stress mediates the cleavage of ATF6 through Site-1 protease (S1P) and Site-2 protease (S2P) to form p50-ATF6, which acts as a transcription factor. Within these current studies, adipocytes treated with Tun induced ATF6 mRNA expression whilst treatment with LPS appeared to lead to a small but significant increase in ATF6 mRNA expression. Furthermore Sal significantly down-regulated ATF6 mRNA expression induced by LPS. Again, in a similar fashion to that observed for SFA, the modest response may relate to the time points investigated, whilst LPS may have a potent effect early on in mRNA expression this may have been lost post 24 hrs treatment.

Grp78/Bip expression appeared unregulated by LPS alone or in combination with any treatment. Despite considerable activation of the UPR, LPS did not induce Grp78/Bip expression. This is of note as previous work has suggested that PERK is found in a complex with Grp78/Bip in cells without either ER stress or in an inactive state (Bertolotti *et al.*, 2000; Zhang *et al.*, 2009). However, under LPS conditions, whilst Grp78/Bip expression appeared unregulated down-stream eIF2 α and CHOP was upregulated in the presence of LPS. It therefore seems likely, as noted for hyperglycaemic conditions, that another mechanism exists for PERK regulation in LPS conditions. The present study also investigated whether these UPR inducing factors would have an effect on p-AktSer473 levels under these present conditions. The findings demonstrated that p-AktSer473 was significantly altered by LPS, in a similar fashion to glucose.

Finally the down-stream effects on adipokine secretion were analysed. The IL-6 secretion data, in response to LPS, were compared with control as well as SFA, glucose and Tun. Interestingly, it appeared that LPS mediated a substantial release of IL-6, and appeared more potent than Tun and glucose, whilst SFA appeared to produce the most potent induction

of IL-6 secretion. Analysis of leptin secretion indicated that LPS appeared to have no apparent effect on the production of leptin from the cultured cells, compared with control or other treatments. These findings appear consistent with previous work examining release of IL-6 and leptin amongst other cytokines (Ryan *et al.*, 2010; Wang *et al.*, 2005). Further examination of IL-6 response with LPS alone, and in combination with chemical chaperones PBA and TUDCA, noted that all treatment regimens increased IL-6 release with the most substantial increase noted with either LPS alone or in combination with PBA. In contrast, leptin release following treatment with LPS caused no apparent change in leptin secretion, whilst LPS in combination with PBA appeared to reduce leptin secretion. Again, as discussed in chapter 5, leptin may serve as a chronic pro-inflammatory agent rather than an acute one, as observed with IL-6.

In summary, LPS had a clear impact on ER stress. The role of LPS may have clinical importance as LPS (endotoxin) is known to have a strong affinity for chylomicrons which are lipoprotein and support LPS to cross the GI mucosa and enter the systemic circulation (Ghoshal *et al.*, 2009). Therefore the insult possessed by the combined effect of LPS and lipoprotein could lead to a dramatic increase in the ER stress pathways within human AT. In addition, as SFA appeared to have a more acute effect on p-AktSer473 and ATF6 mRNA expression than LPS, then both together may increase the ER stress pathways in an acute and chronic fashion. This study further highlights the importance of LPS in its activation of inflammatory pathways and the further need to examine the role of metabolic endotoxaemia in greater detail.

Chapter 7

Final Discussion

7.1 Discussion

Several studies to date have examined the importance of endoplasmic reticulum (ER) stress and its role as a contributor to insulin resistance. Much of this previous analysis has focussed on rodent models of obesity (Ozcan *et al*, 2004; Ozcan *et al*, 2006; Gregor & Hotamisligil, 2007; Hotamisligil, 2008; Sreejayan *et al*, 2008; Xu *et al*, 2010; Yoshiuchi *et al*, 2009; Aguirre *et al*, 2000).

However due to the often noted disparity in functionality of cellular systems between rodents and man, co-current studies have considered the effect of ER stress within human tissues (Boden & Merali, 2011; Deldicque *et al*, 2010; Kars *et al*, 2010). Furthermore studies have specifically examined the role of adipose tissue as the importance of ER stress in obesity associated cellular dysfunction becomes more evident (Miranda *et al*, 2010; Boden *et al*, 2010; van der Kallen *et al*, 2009; Gregor *et al*, 2009). The importance of the ER is clear as it is central to the synthesis, folding and trafficking of secretory and membrane proteins, as such the disruption of this mechanism results in an adaptive unfolded protein response (UPR). The role of UPR is to restore the disrupted ER homeostasis and reduce stress within the system. ER stress has multiple functional effects on many cellular systems that include proliferation, apoptosis, differentiation, glucose homeostasis, as well as insulin signalling (van der Kallen *et al*, 2009; Sreejayan *et al*, 2008; Ozcan *et al*, 2004). With regard to our knowledge of the effect of ER stress within rodent models of obesity, we understand that increasing ER stress leads to impaired insulin action in obese mice (Ozcan *et al*, 2004) ER stress is also increased in obese, insulin resistant individuals (Boden & Merali, 2011) and reduced in tissues of obese subjects post weight loss, in conjunction with improved insulin sensitivity.

However whilst there have been limited studies examining either *ex vivo* adipose tissue or murine adipocyte cells lines, no work has examined mediators of ER stress and the ER stress pathway in human differentiated adipocytes, in detail. As such the studies within this thesis sought to examine the impact of glucose and lipids on the ER pathways, due to their known effects as mediators of IR and the effect of lipopolysaccharide (LPS) - also referred to as endotoxin. In previous cellular studies, endotoxin has been shown to represent a potent stimulus of inflammatory cellular response. Furthermore recent studies with endotoxin have indicated that chronic low levels of circulating endotoxin are increased in subjects with diabetes. As such, these low levels can activate the innate immune pathway within adipose tissue to mediate an inflammatory response affecting insulin sensitivity, combined with the induction of ER stress (Lira *et al*, 2010; Creely *et al*, 2007; Al-Attas *et al*, 2009). In addition to examining ER stress we were keen to also examine particular agents known to reduce ER stress, such as the chemical chaperones 4-phenyl butyric acid (PBA), tauroursodeoxycholic acid (TUDCA) as well as high doses of salicylates (Ozcan *et al*, 2009; Kars *et al*, 2010; Manrique *et al*, 2008; Goldfine *et al*, 2008).

The use of salicylates in patients with T2DM, dates back more than 80 years, with its use gaining a resurgence in recent times (Manrique *et al*, 2008; Goldfine *et al*, 2008) due to a better understanding of some of the original concerns of Salicylate, such as it causing tinnitus as well as gastric bleeding (Croft *et al*, 1971; Fernández *et al*, 1995). As a result, modifications to negate these side effects may lead to a more useful therapeutic agent, as salicylate is now known to impact on the inflammatory regulatory molecule, NFκB resulting in reduced hyperglycaemia (Baron, 1982) and improved insulin signaling, accompanied by an improvement in insulin sensitivity (Yuan *et al*, 2001; Kopp & Ghosh, 1994; Pierce *et al*, 1996).

It should be noted that TUDCA is a bile acid derivative that has been used in Europe to treat cholelithiasis and cholestatic liver disease. Recent studies have shown that TUDCA can improve liver and muscle insulin sensitivity in contrast to its effect in adipose tissue (Kars *et al*, 2010). As such, whilst TUDCA is considered to reduce ER stress, its functions in adipose tissue may in part induce insulin insensitivity, although there is not sufficient data to evaluate this thoroughly (Kars *et al*, 2010). Therefore for this thesis it was clearly relevant to understand the mechanisms by which TUDCA and other chemical chaperones and ER stress alleviating agents may act within human differentiated adipocytes.

Initial mRNA analysis of ER stress within human adipose tissue highlighted that weight gain, as noted through a cross-sectional study examining adipose tissue from lean and obese individuals, increased ER stress. This confirmed and extended previous studies in this area (Boden *et al*, 2008; Gregor *et al*, 2009; Sharma *et al*, 2008). Once the initial changes in ER stress in *ex vivo* human adipose tissue were observed, studies characterised the human pre-adipocyte abdominal subcutaneous differentiation model that would be subsequently used to undertake the *in vitro* ER stress studies. For these studies it was essential to ensure that the pre-adipocyte cells used were fully differentiated, therefore studies were undertaken to characterise the cells using a variety of methods to confirm differentiation had occurred. The characterisation of the cells included examination of changes in mRNA and protein levels during differentiation, using previous studies to affirm our findings. Further analysis was undertaken through assessment of the accumulation of lipid through oil-red O staining as well as lipolysis and lipogenesis data and photographic evidence, as noted over time (details are noted in chapter 4).

Once the differentiated adipocytes were fully characterised, the differentiated cells were treated with LPS, Tun, high glucose (HG) and saturated fatty acids (SFA) with and without salicylate. As discussed in the individual chapters, there were subtle variations in the

impact on ER stress across the pathways and this also affected down-stream adipokine release. However the data demonstrated that LPS, Tun, HG and SFA activated the PERK pathway. PERK is known to be activated in response to the accumulation of misfolded proteins in the ER, and down-stream from PERK the rate of eIF2 α phosphorylation inhibits overall translation to alleviate stress on ER (Harding *et al*, 2000a; Harding *et al*, 2000b). This subsequently induced protein chaperone transcription; however addition of salicylate reversed the PERK activation and reduced pro-inflammatory adipokine release.

Studies also examined the ATF6 pathway which also represents an important ER stress pathway. The studies showed that in adipocytes treated with Tun, ATF6 mRNA expression was significantly induced. Furthermore that endotoxin and high glucose conditions also induced ATF6 mRNA expression, whilst SFA did not significantly induce expression. Salicylate significantly down-regulated the ATF6 mRNA expression induced by LPS, Tun and HG. Taken together, these studies have highlighted that Salicylate could alleviate ER stress by down-regulating the PERK as well as ATF6 pathways and down-stream adipokine signalling.

The present thesis also examined whether UPR inducing factors would have an effect on p-AktSer473 levels. Interestingly, p-AktSer473 was significantly induced by LPS, Tun and HG while SFA had no effect. Further studies with salicylate treating the differentiated cells with either Sal alone or in combination with LPS, Tun or HG highlighted a significant increase in p-AktSer473 activation at least two fold higher than LPS or HG alone. Such a dramatic rise may have arisen due to the combined effects of Salicylate, as an anti-inflammatory, and the insulin sensitising effects of salicylates, where it inhibits the activation of NF κ B via inhibition of phosphorylation and subsequent degradation of I κ B α . Within this thesis it could be deduced that Salicylate has alleviating effects on at least two of the three ER stress pathways, PERK and the ATF6. As such it indicates that, in conditions of obesity,

salicylate has the capacity to reduce protein misfolding which could have beneficial effects on insulin signalling and insulin sensitivity. It should be stressed that, whilst salicylate may provide a 'proof of concept' that it can reduce ER stress, current therapeutic side effects in conditions such as diabetes reduces its capacity for use. However researchers are currently re-evaluating salicylate derivatives as potential new agents in the role to prevent the progression and, in part, reverse the effects of type 2 diabetes as well as vascular disorders (Goldfine *et al*, 2010; Kim, 2010; McCarty, 2010).

7.2 Future Directions

This thesis highlights the relevance of understanding ER stress and its response to various insults in human adipose tissue. Further that ER stress represents a complex, but important metabolic pathway which feeds into the inflammatory response exacerbated in subjects with obesity and T2DM. The current thesis undertook novel work establishing that human differentiated adipocytes, without previous pathological history, would react to insults experienced in the obese and diabetic states. As such future work which would fall beyond the current remit of these studies could examine human differentiated adipocytes, under the same treatment regimens, taken from subjects with and without diabetes as well as those with and without obesity. We already know from *ex vivo* studies that there are changes with ER stress pathways but we are unaware of whether hyperlipidaemia, hyperglycaemia or metabolic endotoxaemia have chronic effects on adipose tissue from patients with obesity or T2DM. It would therefore be interesting to ascertain whether intermittent insults, as might be perceived postprandially, may lead to more ER stress than continual chronic treatment, as previous studies examining the effects to SFA and glucose in human mature adipocytes in chronic and intermittent conditions have shown (Harte *et al*, 2010). This could also examine

whether postprandial influences may represent an important factor in short term changes in the ER mechanism. Based on the collated data within this thesis, it is clear that future studies should look at the role of the IRE1 α pathway by the inducers used within this thesis as well as the effects of salicylates on this pathway. It would also be interesting to understand the mechanism involving Grp78/Bip, under hyperglycaemic conditions, as well as the role of salicylates as a suppressor of ER stress. It is also apparent that the interaction between ER stress pathways and inflammatory pathways are not clearly understood. As such the use of inhibitors as well as knockdown studies in particular pathways may be able to examine the interaction between the ER, NF κ B and JNK and the resulting down-stream effects with and without salicylates. This, in turn, may allow us to undertake detailed analysis on the interaction between ER stress and inflammation and how they may be inextricably linked in conditions of obesity and diabetes.

7.3. Conclusion

This current thesis has sought to expand our knowledge and understanding of the actions of several mediators of insulin resistance. The current studies have highlighted the potential activation of the ER stress pathway through intracellular signalling and insulin pathways, which may combine to alter adipokine release and lead to further dysregulation. Furthermore, comprehension of different metabolic states, such as obesity and diabetes, may require a more complex understanding of the actions and influence of ER stress than has been previously considered. It seems apparent that the peripheral nature and site of action of ER stress necessitates much additional research in order to fully characterise the summative effects of this intriguing organelle disruption upon the human metabolic state.

APPENDICES

APPENDIX I : Solutions and Buffers

AI.1 WESTERN BLOTTING SOLUTIONS

1.1 Sodium Dodecyl Sulphate (SDS) (4%)

10 ml 20% SDS solution

50 ml dH₂O

Solution stored at room temperature (RT)

1.2 Loading buffer

625 µl Tris-HCl (pH 6.8) 125 mM

500 µl SDS 4%

1 ml Glycerol

200 µl Dithiothreitol (DTT)

125 µl Bromophenol Blue

250 µl Distilled H₂O

Electrode Buffer for SDS-PAGE Electrophoresis		
REAGENT	FINAL CONCENTRATION (X5)	QUANTITY (DILUTED IN 1L)
Tris	1.24×10^{-1} M	15 g
Glycine (Biorad, Hercules, CA, USA)	9.6×10^{-1} M	72 g
SDS	20% (v/v)	25 ml

1.3 Transfer Buffer

Transfer Buffer for Electrophoretic Transfer		
REAGENT	FINAL CONCENTRATION (1X)	Quantity (DILUTED IN 4L)
Tris	25 mM	15.15 g
Glycine	192 mM	72.0 g
Methanol	100%	1 L

1.4 Phosphate Buffered Saline (PBS) (pH 7.6)

PBS 120 mM

NaCl 2.7 mM

KCL, 10 mM

1.5 PBS-Tween (PBS-T) (1.0%)

1 L PBS (prepared as above)

1 ml Phosphate Buffered Saline (PBS) ('Tween 20' (0.1% (v/v), Sigma UK).

Solution stored at RT.

1.6 PBS/PBS-T solution for antibody preparation (0.5%)

X quantity 1.0% PBS-T (prepared as above)

X quantity PBS (prepared as above)

1.7 Tris-buffered Saline-Tween (TBS-T) (10X): 0.5M Tris Base, 9% NaCl, pH 7.6

61 g Trizma base

90 g NaCl

1 L dH₂O

Solution mixed to dissolve and pH adjusted using HCl. Solution stored at RT.

1.8 TBS-T (1X)

TBS-T (10X) diluted (1:10) with dH₂O

1.9 Blocking Solution for Millipore® filters (20%)

20 g non-fat milk solution (Marvel Milk Powder, UK)

200 ml PBS 0.5% PBS (Tween 20 (0.1% (v/v), Sigma UK)

AI. 2 GENERAL CELL-CULTURE SOLUTIONS

2.1 Lysis buffer

Ammonium Chloride (NH₄Cl) 0.154 mol/l

Potassium Bicarbonate (KHCO₃) 10 mmol/l

2.2 Collagenase:

50mls of Hank's Buffer Salt Solution

450mls of dH₂O

5mls Pen/Strep

Stored at -20 °C

2.3 Phenol red-free medium

Dulbecco's minimal essential medium (DMEM/F-12) Phenol red free

1% transferrin (see below)

Penicillin (100 U/ml) and streptomycin (100 mg/ml) added.

Medium was stored at 4°C.

2.4 Transferrin

Transferrin binding-protein found in serum, is responsible for the transfer of iron to cells. Transferrin binds Fe^{2+} and prevents its oxidization to Fe^{3+} , preventing loss of iron from the medium. The stimulatory activity of transferrin is proposed to be associated with its iron binding properties and, it is further capable of binding other metal ions in the medium at concentrations which are toxic (Barnes *et al.* 1980).

AI. 3 BUFFERS & SOLUTIONS USED IN RT-PCR PROCEDURES

3.1 DNase Treatment

DNase I	REACTION BUFFER	STOP SOLUTION
1 U/ μl in 50% Glycerol 10 mM Tris-HCl (pH7.5) 10 mM CaCl_2 10 mM MgCl_2	200 mM Tris-HCl (pH 8.3) 20 mM MgCl_2	50 mM EDTA

3.2 Reverse Transcription Buffer

100 mM Tris-HCl (pH 9.0 at 25°C)

500 mM KCl

1% Triton® X-100

APPENDIX II: Reverse Transcription (RT) & Quantitative Real-Time Polymerase Chain Reaction

AII.1 mRNA.

Deoxyribonucleic acid (DNA) or genetic code within a gene encoding the protein structure can be divided into a variable number of introns (non-coding regions) and exons (coding regions), depending on the particular gene. RNA polymerase transcribes the nuclear DNA, synthesising messenger ribonucleic acid (mRNA), with any introns removed. It is these coding regions that ultimately act as ‘blueprints’, determining the configuration and assembly of the specific polypeptide chain that will be assembled within the ribosomes in the cell cytoplasm. Analysis of mRNA expression, allows for estimations to be made as to the level of protein expression of a particular gene of interest

AII.2 RT-PCR.

To carry out a PCR, complimentary cDNA is essential, as it serves as a DNA template for RNA. This is termed ‘reverse transcription’ and subsequently allows the synthesis of cDNA. Such a process involves ‘reading’ mRNA sequences to then assemble a cDNA chain, that comprises the complimentary bases to that on the mRNA strand (guanine bonds with cytosine and, adenine to thymine) (**Figure AII.2.1**).

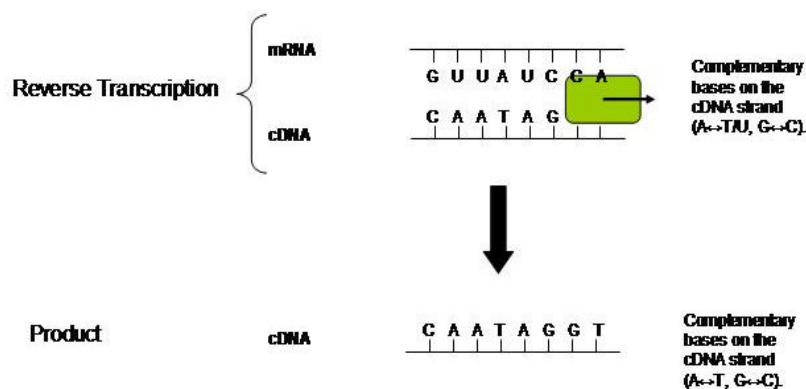


Figure AII.2.1 Diagram illustrating mRNA, which is then transcribed in a reverse transcription process to yield single strands of cDNA. G, guanine; U, uracil; A, adenine; C, cytosine; T, thymine.

AII.3 Quantitative Real-Time PCR.

Quantitative Real-time PCR uses fluorescence technology to monitor amplicon production during each PCR cycle (for instance, in real-time). This allows analysis of the amount of template rather than the amount of amplified product at the endpoint of the reaction. In this study, the mRNA levels were analysed using an ABI 7700 Sequence Detection system, which utilises TaqMan chemistry for highly accurate quantification of specific mRNA levels (**Figure AII.3.1**). TaqMan probes contain a fluorescent reporter dye usually on the 5' base and a quenching dye typically on the 3' base, which suppresses the reporter. Due to the close proximity of the two, the quencher prevents emission of any fluorescence while the probe is intact. If the gene of interest is present, the probe anneals between the forward and reverse primer sites within the PCR product. When the Taq DNA polymerase replicates a template on which a TaqMan probe is bound, its 5' exonuclease activity cleaves the probe. This removes the reporter from the proximity of the quencher, resulting in a fluorescent signal that accumulates with each cycle. The fluorescent signal yielded can be

quantitatively measured by a laser and charged coupled device (CCD) camera, enabling real time detection of cDNA amplification.

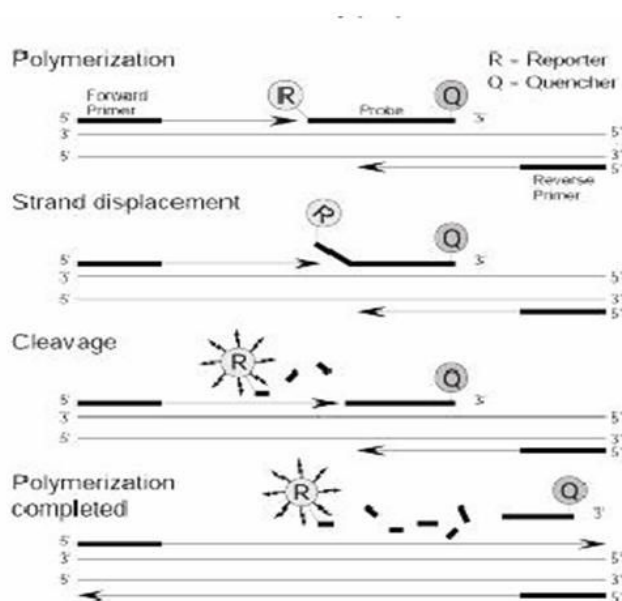


Figure AII.3.1 Diagram outlining TaqMan Sequence Detection Chemistry.

The probe, containing both the fluorescent reporter and quencher dyes, attached to the complimentary target sequence on the DNA. During polymerisation, the reporter dye is cleaved from the probe, allowing increased fluorescence of the reporter. This fluorescence increases in intensity following each amplification cycle, thus making it possible to monitor the reaction in *real-time*.

APPENDIX III: Western Blotting

AIII.1 Calculation of Protein Content of Samples for Western Blot Analysis.

Protein samples were analysed using a spectrophotometer at 655 nm. Conversion of optical densities to protein content (μg) was calculated by the construction of a standard curve (**Figure AIII.1.1**) using bovine serum albumin (BSA) diluted in dH_2O , each time samples were assayed. No protein signal (optical density) was detected in a mixture containing only Reagent S, Reagent A and Solution B, therefore excluding interference with calculated protein sample concentrations.

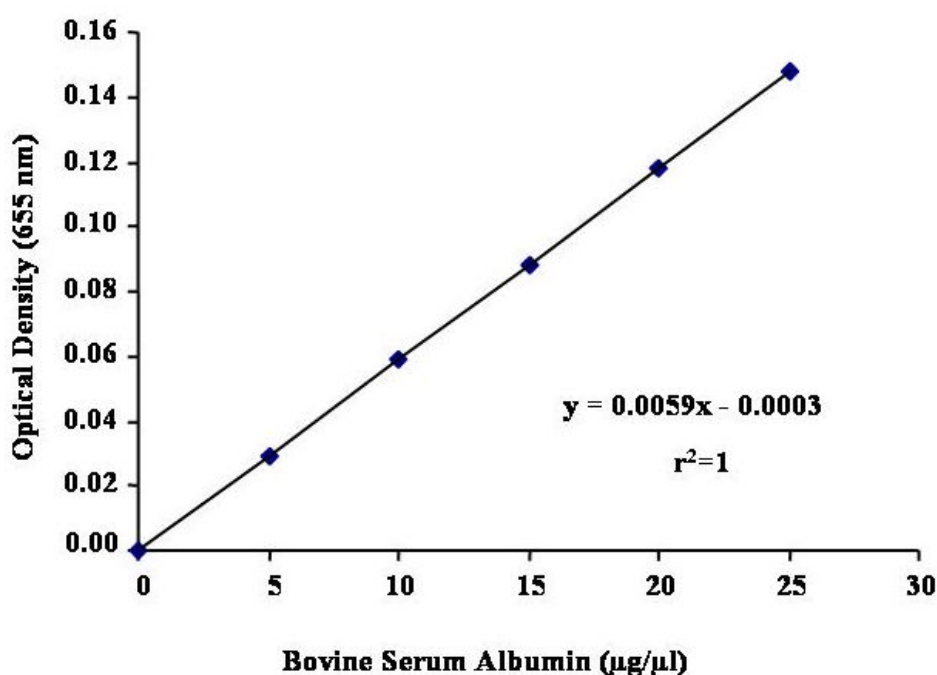


Figure AIII.1.1 Graph to show standard curve used as a reference to calculate protein content in samples of proteins extracted from adipose tissue and isolated adipocytes. Bovine serum albumin was diluted in dH_2O to known concentrations and absorbance read at 655 nm on a spectrophotometer.

AIII.2 Rainbow Marker for Protein Size Comparison in Western Blot Analysis.

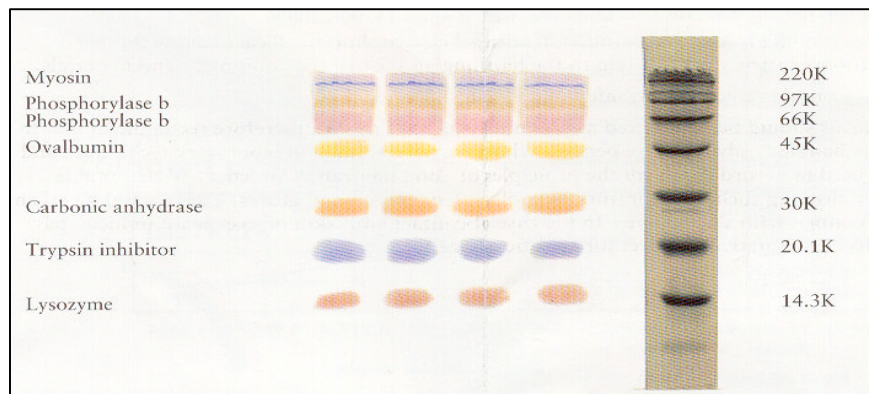


Figure AIII.2.1 A ladder of proteins used as a marker (Amersham Pharmacia Biotech, Buckinghamshire, UK), which is resolved on a 8-15% SDS-PAGE gel (Laemmli 1970). Black bands represent the same gel exposed to photographic film (Hyperfilm™ β -max).

Appendix IV ELISA Methodology

AIV.1 Principle of the Assay

This assay employs the quantitative sandwich enzyme immunoassay technique detailed as follows. A monoclonal antibody specific for the protein of choice (*eg* IL-6 Leptin, Adiponectin) is pre-coated onto a microplate, in this case Leptin for instance. Standards and samples are pipetted into the wells and any 'Leptin' present is bound by the immobilized antibody. After washing away any unbound substances, an enzyme-linked monoclonal antibody specific for leptin is added to the wells. Following a wash to remove any unbound antibody-enzyme reagent, a substrate solution is added to the wells and color develops in proportion to the amount of bound leptin. The color development is stopped and the intensity of the color is measured.

IV.1.1 Calculation of ELISA Results.

Absorbance readings were taken of all wells in duplicates then averaged. The average readings were subtracted from the blank standard optical density.

Then a standard curve graph was constructed by plotting the mean absorbance for each standard on the y-axis against the concentration on the x-axis and draws a best fit curve through the points on the graph. The data may be linearized by plotting the log of the leptin concentrations versus the log of the O.D. and the best fit line can be determined by regression analysis in a similar way to the protein concentration graph undertaken for western blot as outlined in section AIII.1 above. If samples have been diluted, the concentration read from the standard curve must be multiplied by the dilution factor.

Abstracts

1. S Alhusaini, G Tripathi K McGee, K Lois, A Harte, S Kumar (2010) Salicylate induces insulin sensitivity by alleviating Endoplasmic Reticulum (ER) stress in cultured primary human adipocytes. Society for Endocrinology BES 2010. Manchester, UK, 15-18 March 2010.
2. S Alhusaini, K McGee, K Lois, AL Harte, S Kumar, G Tripathi (2010). Effect of Salicylate, lipopolysaccharide and high glucose on Endoplasmic Reticulum (ER) stress pathways in cultured primary human adipocytes. American Diabetes Association, Orlando, USA.
3. SA Alhusaini, K Lois, S Kumar, G.Tripathi (2009). Endoplasmic reticulum stress is induced by lipopolysaccharides and high glucose and is alleviated by salicylates in cultured primary human adipocytes.European Association for the Study of Diabetes, Vienna September. Diabetologia 52(suppl1):S51 **Oral Presentation**

Publication

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Lipopolysaccharide, high glucose and saturated fatty acids induce endoplasmic reticulum stress in cultured primary human adipocytes: Salicylate alleviates this stress

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ABSTRACT

Recent findings indicate that endoplasmic reticulum (ER) stress is significantly increased in adipose tissue of obese human subjects and is critical to the initiation and integration of pathways of inflammation and insulin action. But the factors inducing ER stress in human adipose tissue are unknown. The common factors increased in obesity and linked to insulin resistance are hyperglycaemia, hyperlipidemia and also endotoxemia. Therefore, our aims were to investigate: (1) the role of lipopolysaccharide (LPS), high glucose (HG) and saturated fatty acids (SFA) as inducers of ER stress in primary human adipocytes and (2) whether salicylate, a known anti-inflammatory compound, can alleviate this effect. Components of the ER stress pathways were studied in human abdominal subcutaneous (AbSc) adipose tissue (AT) from obese and lean. Following the culture and differentiation of primary human preadipocytes, these adipocytes were treated with LPS, HG, tunicamycin (Tun) and SFA either alone or in combination with sodium salicylate (Sal). Markers of ER stress were significantly increased in AbSc AT of obese. Differentiated human adipocytes treated with LPS, Tun, HG and SFA showed significant activation of eukaryotic translation initiation factor 2 α (eIF2 α) and activating transcription factor 6 (ATF6) and their down-stream targets. Sal alleviated this effect and activated AktSer473 phosphorylation. This study presents important evidence that: (1) there is increased ER stress in adipose tissue of obese individuals, (2) LPS, hyperglycaemia and saturated fatty acids induce significant ER stress in primary human adipocytes and (3) this induction is alleviated by salicylate.

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1. Introduction

Obesity-associated inflammation is a key contributory factor in the pathogenesis of type 2 diabetes mellitus (T2D) and cardiovascular disease (CVD) but the fundamental mechanisms responsible for activating innate immune inflammatory pathways and insulin resistance are currently unclear. Murine studies have revealed that one key link is increased endoplasmic reticulum (ER) stress [1]. The ER has a central role in lipid and protein biosynthesis. During pathological nutrient excess, proteins formed in the ER may fail to attain correct conformation and accumulation of misfolded proteins in the ER causes stress and activates the Unfolded Protein Response (UPR) signal [2].

The UPR signals through three ER transmembrane sensors: PKR-like ER-regulated kinase (PERK), inositol requiring enzyme1 α

(IRE1 α) and activating transcription factor 6 (ATF6) [3]. These activate an adaptive response that results in inhibition of protein translation and increase in transcription of protein-folding chaperones and ER-associated degradation genes [2,4]. PERK phosphorylates the eukaryotic translation initiation factor 2 α (p-eIF2 α) [5]. p-eIF2 α then attenuates protein synthesis and reduces ER protein overload and also activates activation transcription factor4 (ATF4), which induces expression of many genes, including those involved in apoptosis: C/EBP homologous protein (CHOP) [6]. ATF6 activates transcription of ER chaperones: glucose regulated protein (Grp)78/Bip, protein disulfide isomerase (PDI), Ero1-L α and calnexin to augment the protein folding capacity [1].

An enhanced level of the UPR has been demonstrated in obese, insulin-resistant human adipose tissues [7–9]. ER stress and the UPR are linked to major inflammatory and stress-signalling networks, including the activation of JNK and IKK-NF κ B pathways which play a central role in obesity-induced inflammation and metabolic abnormalities [10]. High doses of salicylates have been shown to lower blood glucose concentrations [11]. Severely obese

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rodents when treated with salicylates demonstrated reduced signalling through IKK β pathway and this was accompanied by improved insulin sensitivity *in vivo* [12,13].

Although ER stress and metabolic dysfunction is associated with obesity in rodent models, the importance of ER stress and the potential inducers of ER stress in human adipocytes are not known. Therefore, the objective of the present study was to show the existence of ER stress in obese human adipose tissue, identify the potential originators of this stress and also demonstrate the role of anti-inflammatory agent, sodium salicylate on ER stress in primary human adipocytes. The primary human preadipocytes were cultured and fully differentiated adipocytes were treated with most probable factors: lipopolysaccharides (LPS), high glucose (HG), tunicamycin (Tun) and saturated fatty acids (SFA) with and without salicylate (Sal). ER stress pathways and Akt activation were studied.

2. Materials and methods

2.1. Subjects

Human Abdominal Subcutaneous (AbSc) adipose tissue (AT) was collected from patients (age: 40.8 (mean \pm SD) \pm 5 yrs; Lean BMI: 22.04 \pm 2.6 kg/m² and obese BMI 30 \pm 3.5 kg/m²) undergoing liposuction surgery with informed consent obtained in accordance with LREC guidelines and with ethics committee approval. All tissue samples were flash frozen and/or utilized for isolation of human preadipocytes as detailed [14].

2.2. Cell culture

Human AbSc AT (BMI 25.04 \pm 0.6 kg/m²; n = 3–6) were digested with collagenase to isolate preadipocytes as described by Zuk et al. [14]. Preadipocytes were then cultured into tissue culture flasks until confluent and then trypsinized to obtain cells to carry out the study. The preadipocytes from the same passage were grown in 6-well plates (10⁴ cells/well in 2 ml media) to confluence in DMEM/Ham's F-12 phenol-free medium (Invitrogen, UK) containing 10% FCS, penicillin (100 U/ml), streptomycin (100 μ g/ml), and transferrin (5 μ g/ml). At confluence, preadipocytes were differentiated in differentiation media (Promocell, Germany) containing biotin (8 μ g/ml), insulin (500 ng/ml), Dexamethasone (400 ng/ml), IBMX (44 μ g/ml), L-Thyroxine (9 ng/ml) and Ciglitazone (3 μ g/ml) for 72 h. After this period, the differentiating cells were grown in nutrition media containing (NM) DMEM/Ham's F-12, 3% FCS, D-biotin (8 μ g/ml), insulin (500 ng/ml) and Dexamethasone (400 ng/ml) until fully differentiated (14–18 days). The viability of adipocytes was assessed using the trypan blue dye exclusion method (Sigma–Aldrich) [15].

2.3. Treatments

Differentiated adipocytes (day 15) 24 h prior to treatments were switched to detoxification media (DMEM/Ham's F-12 phenol-free medium containing only 2% serum) to remove effects of growth factors and other components in NM. The treatments were then placed in the fresh detoxification media. The cells were treated with lower or already published concentrations of LPS (100 ng/ml) [16], Tun (750 ng/ml) [2], glucose (HG) (25 mM), SFA (2 mM) and Sal (20 mM) for a maximum of 24 h. SFA was prepared as 40 mM stocks by dissolving Stearic Palmitic acid Mixture (Fluka, UK) in absolute ethanol and then lyophilised and was re-constituted in 1 ml 3% BSA (Free-fatty acid free) in Gey's balanced salt solution (Sigma–Aldrich). The dissolving buffers or solvents were used as controls. All the data shown in this study is from 24 h treatments only, even though for LPS and Tun, lower time points of 6 and 12 h were also studied.

2.4. Immunoblotting

Cells were washed with cold PBS and harvested in lysis buffer (20 mM Tris–HCl, pH 7.5; 137 mM NaCl; 1 mM EGTA, pH 8; 1% Triton X-100; 10% glycerol; 1.5 mM MgCl₂) containing protease and phosphatase inhibitors (10 mM NaF; 1 mM PMSF; 1 mM sodium metavanadate; 5 μ g/ml aprotinin; 10 μ g/ml leupeptin) (Sigma–Aldrich, UK) and stored at -80°C until required. SDS–PAGE and Western blot analysis were performed as described [16]. Primary antibodies were: phospho and total Akt, phospho and total eIF2 α , Bip/GRP78, Calnexin, PDI, Ero1-L α , phospho-PERK (p-PERK), IRE1 α and β -actin (Cell Signalling Technologies). Antigen–antibody complexes were visualized using ECL reagents (Amersham, UK). Autoradiographs were semi-quantified using 2D densitometry software (GeneTools, UK). The bands were first normalised as a function of the loading control (protein of interest/ β -actin) or total expression of the proteins (for phosphor proteins), then converted to fold change compared with controls.

2.5. Extraction of RNA and quantitative RT-PCR

RNA was extracted from adipocytes (RNeasy Lipid Tissue Mini Kit, Qiagen), according to manufacturers' instructions. Following DNase treatment and reverse transcription, mRNA expression levels were determined using an ABI 7500 Real-time PCR Sequence Detection system [17]. Pre-optimized quantitative primer and probe sequences for genes ATF6, Ero1-L α , CHOP and PDI were utilized (Applera, Cheshire, UK). All reactions were multiplexed with the housekeeping gene r18S (Applera). Data were obtained as cycle threshold (Ct) values and used to determine ΔCt values (ΔCt = Ct of gene of interest – Ct of r18S). Measurements were carried out on at least three occasions for each sample. To exclude potential bias due to averaging, data was transformed through the Power equation $2^{-\Delta\Delta\text{Ct}}$.

2.6. Statistical analysis

Data is presented as mean \pm standard error of the mean (SEM) for at least three independent experiments to ensure reproducibility. Students *t* test was used to compare values between two groups unless stated otherwise. *p* Values <0.05 were considered to represent statistically significant differences.

3. Results

3.1. ER stress markers are up-regulated in obese human AbSc AT

Protein expression of the ER stress markers was measured in four obese and four lean human AbSc AT. The p-PERK and IRE1 α proteins were increased in obese subjects – although this was only significant for IRE1 α expression compared with lean (Fig. 1A). ATF6, which regulates the third ER stress pathway, was investigated by examining mRNA expression via real-time PCR, as this proved a more reliable method [18]. ATF6 mRNA expression from AbSc AT of 10 lean and 10 obese subjects showed to be significantly higher (8-fold) in obese subjects (ΔCt = 07.36 \pm 1.47) compared with lean (ΔCt = 10.57 \pm 1.13) (p < 0.001) (Fig. 1B). Furthermore, the protein expression of down-stream targets of the ER stress pathways, Grp78/Bip1, Calnexin, PDI and Ero1-L α , were all significantly increased in AbSc AT of obese subjects (Fig. 1A).

3.2. LPS, Tun, HG and SFA up-regulate PERK and ATF6 pathways in primary human adipocytes: Sal down-regulates them

To determine the origins of ER stress fully differentiated human adipocytes were treated with most probable factors elevated in

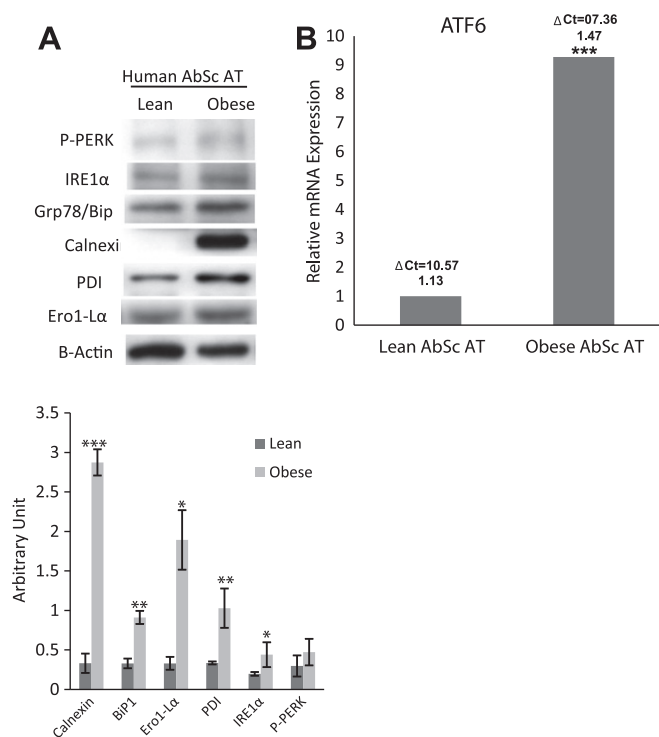


Fig. 1. Expression of ER stress markers. (A) Protein expression levels of ER stress markers: p-PERK, IRE1 α , Grp78/Bip, Calnexin, PDI, Ero1-L α and β -actin (loading control) in lean ($n = 4$) vs. obese ($n = 4$) human AbSc AT. The protein expression was determined from whole AT lysate by Western blot. (B) mRNA expression of ATF6 in human AbSc AT, Lean vs Obese: both $n = 10$. mRNA was determined by qRT-PCR. In the bar figure, values are the mean \pm SEM ($n = 4$). $p < 0.05$, $p < 0.01$ and $p < 0.001$ by Student's t -test.

obesity such as LPS, HG and SFA for 24 h. Tun which is a known inducer of ER stress was used as a positive control. The protein, p-eIF2 α , down-stream-target of p-PERK, was significantly induced by LPS, Tun, HG and SFA compared with cells treated just with the solvent of these substrates (controls) (Fig. 2A and B). Sal significantly down-regulated p-eIF2 α in adipocytes treated with LPS, Tun, HG and SFA in combination with Sal (Fig. 2A and B). There was no change in the total eIF2 α expression levels. This provides first strong evidence that LPS, HG and SFA activate PERK pathway while Sal alleviates this activation in human adipocytes.

The ATF6 mRNA level was significantly increased in the cells treated with LPS, HG and Tun but was not significant in SFA treated cells (Fig. 2C). ATF6 mRNA levels were significantly down-regulated in adipocytes treated with LPS, HG and Tun in combination with Sal and Sal alone (Fig. 2C). Sal also down-regulated ATF6 mRNA expression in SFA treated cells although not significantly (Fig. 2C). LPS and Tun showed significant activation of eIF2 α phosphorylation and ATF6 expression after 6 and 12 h as well and Sal down-regulated them (data not shown).

3.3. Down-stream targets of PERK and ATF6 pathway are up-regulated by LPS, Tun, HG and SFA: Sal alleviates ER stress response

Protein chaperones and down-stream targets of PERK and ATF6 pathways either protein or mRNA expression levels were measured in the adipocytes treated with LPS, Tun, HG, SFA alone or in combination with Sal. Grp78/Bip was significantly increased in Tun and SFA treated cells only (Fig. 3A and B). There was an increase in LPS treated cells but this was not significant. HG did not have any effect on Grp78/Bip expression. Sal significantly down-regulated the expression of Grp78/Bip in Tun and SFA treated cells

(Fig. 3A and B). Another protein chaperone calnexin was significantly up-regulated by LPS, Tun, HG and SFA (Fig. 3C and D) which was significantly down-regulated by Sal (Fig. 3C and D). Sal alone also significantly suppressed the calnexin expression compared to the controls. mRNA expression of protein chaperones, PDI and Ero1-L α were significantly increased in LPS, Tun, HG and SFA treated adipocytes and Sal significantly down-regulated their expressions (Fig. 3E and F). Only PDI wasn't significantly suppressed by Sal in HG treated cells. However, Sal alone significantly down-regulated PDI mRNA expression levels (Fig. 3E).

Signalling through PERK, ATF6 and IRE1 α can induce pro-apoptotic signals by activating down-stream targets such as CHOP. All these treatments significantly increased the CHOP expression (at least 2-folds) (Fig. 3G). Interestingly, Sal significantly down-regulated the CHOP expression induced by LPS, Tun, HG and SFA (Fig. 3G).

3.4. Activation of AktSer473

Hu et al. [19] have reported transient activation of Akt in ER-induced apoptosis and p-Akt (Ser473) is also an important member of insulin signalling pathway as it induces glucose transport and overall insulin sensitivity of the cell. Therefore, Akt activation was examined by measuring p-AktSer473. Interestingly, LPS, Tun and HG significantly induced p-AktSer473 levels compared to controls. There was further significant induction by Sal in adipocytes treated with LPS, Tun, HG and SFA (Fig. 4A and B). Sal alone also significantly increased AktSer473 phosphorylation (Fig. 4A and B).

4. Discussion

The results of the present study demonstrate that firstly, there is increased ER stress in obese human AbSc AT, secondly, the factors inducing this response could be LPS, hyperglycaemia and SFA and thirdly, this stress response is alleviated by salicylates and could contribute to increased insulin sensitivity in adipocytes. LPS [20], hyperglycaemia [21] and free-fatty acids [22] have all been shown to be elevated in blood during obesity and have been linked to increased inflammation and insulin resistance and therefore, were investigated as possible factors for inducing ER stress in human adipocytes. Tunicamycin is a well known ER stress inducer and therefore was used as a positive control.

ER stress protein markers were significantly up-regulated in obese human AbSc AT compared to AbSc AT from lean subjects. The IRE1 α and ATF6 expression levels were significantly increased in obese AbSc AT. The p-PERK was also increased but the increase was not significant. This observation confirms the three previous studies carried out on human adipose tissue [7–9]. The protein chaperones Grp78/Bip, Calnexin, Ero1-L α and PDI were all significantly up-regulated in obese human AbSc AT compared to lean AbSc AT and this confirms the earlier observation [7].

To identify the factors responsible for inducing ER stress in human AT, stromal fractions isolated from human AT were cultured and then differentiated. The differentiated adipocytes were then treated with LPS, Tun, HG and SFA with and without salicylate. Our data clearly demonstrates that LPS, Tun, HG and SFA activate the PERK pathway. This activation was measured by looking at the expression levels of p-eIF2 α , a down-stream target of PERK which was significantly increased in LPS, Tun, HG and SFA treated adipocytes. P-eIF2 α activation was totally eliminated when the above treatments were given in combination with salicylate. This observation contradicts another study in promonocytic cell line THP-1 where salicylate and aspirin have been shown to induce eIF2 α phosphorylation and hence protein synthesis attenuation [23]. It could be a cell specific response. Our observation is further

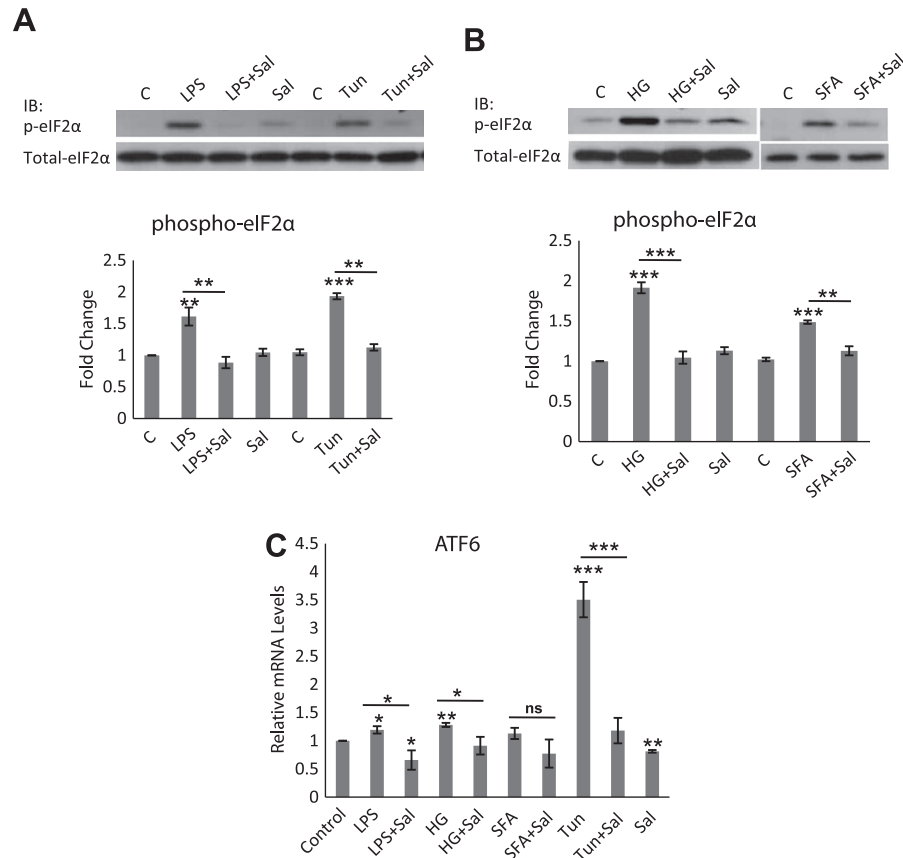


Fig. 2. ER stress pathway expression studies from adipocytes treated with LPS, Tun, HG and SFA either alone or in combination with Sal. (A) p-elf2α in LPS and Tun treated adipocytes either alone or in combination with Sal. (B) p-elf2α in HG and SFA treated adipocytes either alone or in combination with Sal. B and C were measured by Western blot. (C) mRNA expression ATF6 in adipocytes treated with LPS, Tun, HG and SFA either alone or in combination with Sal. mRNA was determined by qRT-PCR. In the bar figure, values are the mean \pm SEM ($n = 3-6$). $p < 0.05$, $** p < 0.01$ and $*** p < 0.001$ by Student's *t*-test.

supported by the significant increase in expression of protein chaperones, specifically CHOP by LPS, Tun, HG and SFA and the induction of which was significantly down-regulated by Sal. It has been shown that to up-regulate CHOP transcription the PERK-elf2α-ATF4 branch of the UPR is essential [24].

Modification of ATF6 protein is important for the ER stress response. Namba et al. [18] have shown that all the ER stressors they tested (such as thapsigargin and tunicamycin) up-regulated ATF6 mRNA expression with the cells over-expressing ATF6 mRNA showing enhanced ER stress response. Indeed the adipocytes treated with tunicamycin, in this study showed highly significant induction of ATF6 mRNA expression and also the down-stream targets of ATF6 compared to the controls. Therefore, up-regulation of ATF6 mRNA expression could be used as an indicator for activation of the ATF6 pathway. LPS and HG also induced ATF6 mRNA expression significantly while SFA did induce some expression but it was not significant. Salicylate significantly down-regulated the ATF6 mRNA expression induced by LPS, Tun and HG. Induction of ATF6 pathway is supported by the increase in expression of protein chaperones up-regulated by ATF6. The protein chaperones calnexin, PDI, Ero1- α and CHOP expression were induced in adipocytes treated with LPS, Tun, HG and SFA. Sal again helps in alleviating ER stress by down-regulating both the PERK as well as ATF6 pathways.

The Grp78/Bip expression was significantly increased only by Tun and SFA which was in turn down-regulated by Sal. LPS did show activation but it was not significant. Despite considerable activation of the UPR, HG did not induce Grp78/Bip expression. Zhang et al. [25] have made the same observations in INS-1 pancreatic β -cells treated with high glucose (30 mM). The reason for this

is not yet known and would be interesting to investigate further because Bertolotti et al. [26] have shown that PERK is found in a complex with Grp78/Bip in cells without ER stress conditions and is inactive. In order to activate elf2α, it must dissociate from Grp78/Bip under UPR condition and our results clearly demonstrate that the p-elf2α and its down-stream target CHOP are activated by HG. It is quite possible that another mechanism exists for PERK activation under hyperglycaemic condition.

The present study also investigated whether these UPR inducing factors would have effect on p-AktSer473 levels. Interestingly, p-AktSer473 was significantly induced by LPS, Tun and HG while SFA had no effect. This is not a unique observation, e.g., Hu et al. [19] have reported transient activation of Akt during ER stress, induced by thapsigargin and tunicamycin in MCF-7 cells. They have also shown blocking Akt activity, sensitised MCF-7 cells to ER stress-induced apoptosis, suggesting that Akt activation is a pro-survival pathway, activated during ER stress. Ho et al. [27] have also shown Akt activation under hyperglycaemic condition (33 mM Glucose) in human umbilical vein endothelial cells (HUVECs) within 24 h. Similar observation has been made in LPS treated THP-1 cells [28]. The adipocytes treated with Sal alone or in combination with LPS, Tun or HG induced p-AktSer473 significantly, at least two fold higher than LPS or HG alone. This could be because of the anti-inflammatory and insulin sensitising effect of salicylates, where it inhibits the activation of nuclear factor- κ B (NF- κ B) via inhibition of phosphorylation and subsequent degradation of I κ B α [13,29].

Salicylate is an interesting molecule and in our study we have demonstrated that it successfully alleviates ER stress induced by LPS, Tun, HG and SFA. From this study we could also deduce that

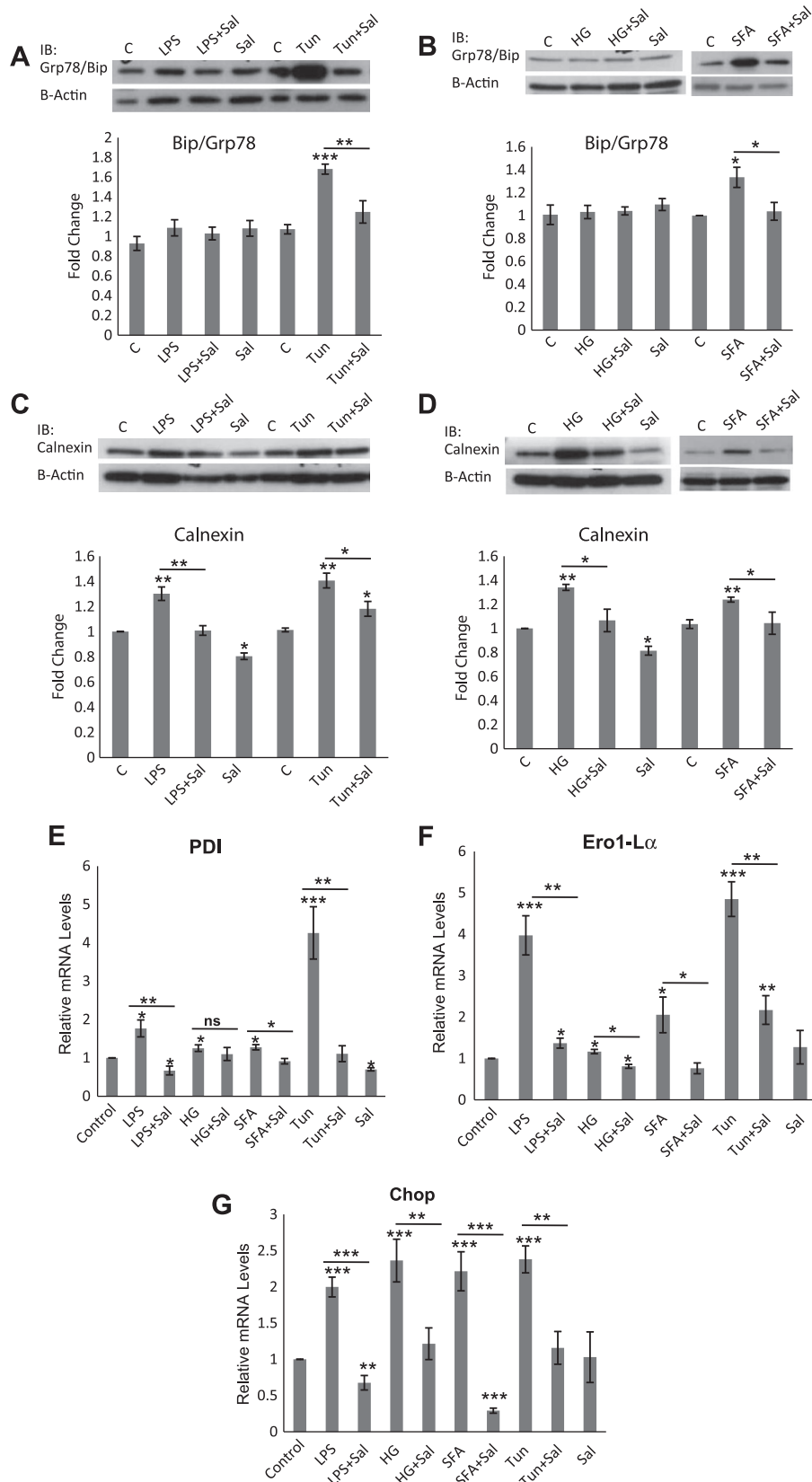


Fig. 3. Expression of ER stress chaperones. (A) Protein expression levels of Grp78/Bip in LPS and Tun treated adipocytes either alone or in combination with Sal. (B) Protein expression levels of Grp78/Bip in HG and SFA treated adipocytes either alone or in combination with Sal. (C) Protein expression levels of Calnexin in LPS and Tun treated adipocytes either alone or in combination with Sal. (D) Protein expression levels of Calnexin in HG and SFA treated adipocytes either alone or in combination with Sal. (E) mRNA expression PDI in adipocytes treated with LPS, Tun, HG and SFA either alone or in combination with Sal. (F) mRNA expression Ero1-L α in adipocytes treated with LPS, Tun, HG and SFA either alone or in combination with Sal. (G) mRNA expression CHOP in adipocytes treated with LPS, Tun, HG and SFA either alone or in combination with Sal. mRNA was determined by qRT-PCR. In the bar figure, values are the mean \pm SEM ($n = 3-6$). $p < 0.05$, $p < 0.01$ and $p < 0.001$ by Student's t -test.

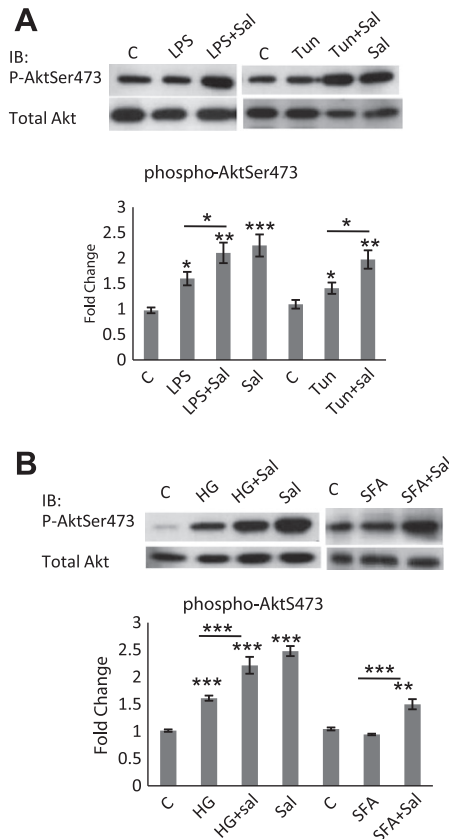


Fig. 4. Expression of p-AktSer473. (A) Protein expression levels of p-AktSer473 in LPS and Tun treated adipocytes either alone or in combination with Sal. (B) Protein expression levels of p-AktSer473 in HG and SFA treated adipocytes either alone or in combination with Sal. Protein expression was measured by Western blot. In the bar figure, values are the mean \pm SEM ($n = 3-6$). $p < 0.05$, $p < 0.01$ and $p < 0.001$ by Student's t -test.

it has alleviating effect on at least two of the three ER stress pathways, namely, the PERK and the ATF6. The mechanism by which salicylate ameliorates ER stress is unknown. One of the possible mechanisms could be the one demonstrated by Yuan et al. [12] where they and others have shown that salicylates can inhibit the activation of NF- κ B by preventing the phosphorylation and subsequent degradation of I κ B α by down-regulating I κ B kinase β (IKK β) [12,30].

In conclusion this manuscript has addressed two main issues: (1) what can induce ER stress in human adipocytes and (2) that salicylate can alleviate the ER stress induced by these factors. We have clearly demonstrated that endotoxemia (LPS), hyperglycemia (HG) and hyperlipidemia (SFA) could independently induce significant ER stress in primary human adipocytes specifically and salicylate alleviated this stress. In addition, salicylate may be inducing insulin sensitivity by activating Akt. Although all of these factors can induce ER stress in adipose tissue the mechanism for inducing ER stress might not be the same and future studies should concentrate on delineating the mechanisms involved. Role of salicylates as a suppressor of ER stress also needs further investigation.

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